Universidade de Aveiro Departamento de Biologia
2014

Bárbara Gomes Magalhães

Evaluation of a new molecular typing strategy of *Pseudomonas aeruginosa*

Avaliação de uma nova estratégia de tipagem molecular de *Pseudomonas aeruginosa*

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(Bárbara Gomes Magalhães)

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Evaluation of a new molecular typing strategy of *Pseudomonas aeruginosa*

Avaliação de uma nova estratégia de tipagem molecular de *Pseudomonas aeruginosa*

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica do Doutor Dominique Stéphane Blanc, Dr. ès Science, Priva Docent, Maître d'enseignement et de recherche, e chefe de laboratório de epidemiologia e de tipagem microbiana do Hospital Universitário de Lausana, e sob a co-orientação do Doutor Artur Jorge da Costa Peixoto Alves, investigador principal do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar (CESAM), da Universidade de Aveiro.

Dedicada à pessoa mais importante da minha vida, a minha mãe.

o júri

Presidente

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Palavras-chave

Pseudomonas aeruginosa, epidemiologia, unidades de cuidado intensivo (UCIs), tipagem molecular, relacão clonal.

Resumo

Pseudomonas aeruginosa é a terceira causa de infeção adquirida em hospitais, em pacientes hospitalizados em unidades de cuidado intensivo (UCIs). Este microrganismo é responsável por um elevado número de doenças nosocomiais, e pelo sua gravidade. Uma vez que é ubíquo no ambiente e também constitiu a microbita endógena de pacientes hospitalizados, existe a necessidade de utilizar métodos de tipagem molecular eficientes no estabelecimento de relações clonais entre isolados. Double Locus Sequence Typing (DLST) tem sido usado recentemente na análise de relações clonais entre estirpes de *P. aeruginosa*, provando ser eficaz, fácil, e reduzindo também o tempo de manipulação e custos de análise. Outra técnica de tipagem chamada Double Digest Sequence Label (DDSL) foi também descrita no estudo molecular deste microrganismo. Um elevado poder discriminatório torna DDSL num complemento putativo à tipagem para resolver clusters de DLST em situações específicas.

De 2010 a 2012 observou-se um aumento da incidência de infeções por *P. aeruginosa* nas UCIs do Hospital Universitário de Lausana, na Suíça. Durante este período, 689 isolados foram recolhidos de 254 pacientes. Todos os isolados foram analisados com DLST e agrupados em 46 DLST clusters, dos quais 4 clusters foram posteriormente investigados neste estudo (cluster 1_18, 1_21, 6_7 e 28_77). Estes 4 clusters foram retrospectivamente tipados com o método DDSL para verificar se se poderia alcançar uma melhor discriminação dos isolados. Para isso, um primeiro passo de optimização de DDSL foi realizado, o qual resultou em perfis de fingerprinting de boa qualidade. Contudo, a análise quantitativa dos resultados usando o software BioNumerics não foi possível. A comparação visual dos perfis de fingerprinting de DDSL para cada cluster permitiu a formação de diferentes tipos de DDSL, mas não a determinação de bandas diferentes entre os mesmos.

Os dados epidemiológicos mostraram que a contaminação de ambientes húmidos provavelmente desempenhou um papel importante na disseminação de estirpes de *P. aeruginosa* neste surto. Comparação de informação epidemiológica e molecular mostrou que a maioria dos tipos de DDSL não distinguíveis estavam epidemiologicamente ligados, levando à suposição de que a transmissão paciente-para-paciente deveria ser altamente considerada, como visto para o cluster 1_18. No entanto, a evolução da estirpe deve ser considerada aquando do estudo de um surto de longa duração.

Concluindo, esta nova estratégia de tipagem de *P. aeruginosa* permitiu obter uma imagem geral acerca da epidemiologia deste surto. Todavia, DDSL é um método tecnicamente complexo, demorado e subjectivo, não eficiente para ser usado para fins de investigação epidemiológica.

Keywords

Pseudomonas aeruginosa, epidemiology, intensive care units (ICUs), molecular typing, clonal relatedness.

Abstract

Pseudomonas aeruginosa is the third leading cause of hospital acquired infection in intensive care unit (ICU) patients. This microorganism holds responsibility in a high number of nosocomial infections and their severity. Because it is ubiquitous in the environment and also constitutes the endogenous microbiota of hospitalized patients, there is a need to use powerful molecular typing methods to establish clonal relationships between individual isolates. Double Locus Sequence Typing (DLST) has recently been used in the analysis of *P. aeruginosa* strains relatedness, proving to be efficient, easy, and also reducing the working time and costs of analysis. Another typing technique called Double Digest Sequence Label (DDSL) had also been reported in the molecular study of this microorganism. A higher discriminatory power makes DDSL a putative typing complement to resolve DLST clusters in specific situations.

From 2010 to 2012, an increase in *P. aeruginosa* infections incidence was observed in the ICUs of the Lausanne University Hospital, Switzerland. During this period, 689 isolates were retrieved from 254 patients. All isolates were analyzed with DLST and grouped in 46 DLST clusters, from which 4 clusters were further investigated in this study (cluster 1_18, 1_21, 6_7 and 28_77). These 4 clusters were retrospectively typed with the DDSL method to verify if an improved discrimination of isolates could be achieved. To do so, a first DDSL optimization step was performed, which resulted in good quality fingerprinting profiles. However, a quantitative analysis of the results using BioNumerics software was not possible. Visual comparison of DDSL fingerprinting patterns within each cluster allowed the formation of different DDSL types, but not the determination of bands differences between them.

Epidemiological data showed that contamination of humid environments probably played an important role in the dissemination of *P. aeruginosa* strains in this outbreak. Comparison of epidemiological and molecular information showed that most of undistinguishable DDSL types were epidemiologically linked, leading to the assumption that patient-to-patient transmission should be highly suspected, as seen for cluster 1_18. Nevertheless, strain evolution should be considered when studying a long period outbreak.

In conclusion, this new typing strategy of *P. aeruginosa* allowed the acquisition of a general picture about this outbreak's epidemiology. Nevertheless, the DDSL is a technically complex, time consuming and subjective technique, not efficient to be use for epidemiological investigation purposes.

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List of abbreviations

- **BCIP –** 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
- **Bio-14-dCTP –** dCTP with biotin attached at the N^4-position of the pyrimidine base by a 14-atom linker
- **BSA –** Bovin serum albumin
- **CF –** cystic fibrosis
- **dCTP –** Deoxycytidine triphosphate
- **DDSL** – Double digest selective label
- **DLST** – Double locus sequence typing
- **DNA** – Deoxyribonucleic acid
- **EPS** – Extracellular polymeric substances
- **ICU** – Intensive care unit
- **LB** – Luria-Bertani broth
- **LPS** – Lipopolysacharide
- **MLST –** Multilocus sequence typing
- **MLVA** - Multiple-Locus Variable number tandem repeat Analysis
- **MRPA –** Multi-Resistant *Pseudomonas aeruginosa.*
- **NBT** – Nitro-blue tetrazolium chloride
- **NGS** – Next generation sequencing
- **PCR –** Polymerase chain reaction
- **PFGE** – Pulsed-field gel electrophoresis
- **QS** – Quorum sensing
- **RAPD –** Randomly Amplified Polymorphic DNA-PCR
- **REP - PCR** – Repetitive sequence-based PCR
- **RFLP –** Restriction fragment length polymorphism
- **Rpm** – Revolutions per minute
- **rRNA** – Ribosomal ribonucleic acid
- ***Sty*I-HF** – High fidelity restriction endonuclease *Sty*I

- **T3SS** – Type 3 Secretion System
- **TAE** – Tris-acetate-EDTA
- **UTI** – Urinary tract infection
- **UV** – Ultraviolet
- **VNTR** – Variable number of tandem repeat
- **WGS** – Whole genome sequencing

I. Introduction

1. The genus *Pseudomonas*

Members of the genus *Pseudomonas* are gram-negative, non-spore-forming bacilli that are 1.5 to 5 µm long and 0.5 to 1.0 µm wide. *Pseudomonas* subsp. are motile due to the presence of one or more polar flagella (Holt et al. 1994) (Figure 1). They possess a respiratory metabolism with oxygen as the terminal electron acceptor. However, some isolates can grow under anaerobic conditions by using nitrate or arginine as the terminal electron acceptor (Stanier *et al.* 1966). They display a remarkable metabolic versatility, which allow them to colonize very different ecological niches, from the environment to the interaction with different eukaryotic hosts (Spiers *et al.* 2000). They are found in water and soil, and on plants, including fruits and vegetables (Silby et al. 2011) Some species are well recognized as pathogens of plants or animals, like *P. syringae* or *P. aeruginosa* (He et al. 2004; Lindeberg et al. 2009). Other species, like *P. fluorescens*, colonize and develop beneficial interactions with roots of plants (Preston et al. 2001).

Figure 1. Illustration of *P. aeruginosa* microcolonies, polar pili, and flagellum. Figure was achieved through a scanning electron photomicrograph (Tredget et al. 2004).

2. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is the best-known and studied member of the genus *Pseudomonas*. Besides colonizing humans, animals and plants, it is a ubiquitous organism, highly disseminated through the environment, mostly in moist and wet niches (Blanc et

al. 2007). Nevertheless, *P. aeruginosa* remains mainly known for its importance in the medical field, being one of the most important nosocomial pathogens. The first report of *P. aeruginosa* in a human infection was probably described by Luke in 1862 (Palleroni 2010). He observed the presence of rod-shaped particles in pus of infections that possessed blue-green coloration, which was similar to the one already observed by Sedillot on surgical dressings (Palleroni 2010). Nowadays, it is known that this coloration is due to pyocyanin and other pigments produced by *P. aeruginosa*. The first isolation from infections was done in 1882 by Gessard, who identified it as *Bacillus pyocyaneus* (Lyczak et al. 2000).

2.1. Metabolism

In general, *Pseudomonas* species possess remarkable nutritional versatility. For instance, one strain of *Pseudomonas multivorans* has been shown to use up to 108 diverse organic compounds as carbon source (Stanier et al. 1966). In the case of *P. aeruginosa*, a variety of carbon/energy sources are exploited for growth, such as carbohydrates, amino acids, fatty acids and, by preference, tricarboxylic acid intermediates, like malate, fumarate, and succinate (Ornston 1971; Valentini et al. 2011) Additionally, *P. aeruginosa* can also perform reduction of nitrogen-containing compounds (Sias et al. 1980)

2.2. *Pseudomonas aeruginosa* genome

In 2000, Stover et al. published the first complete genome sequence of *P. aeruginosa*. This discovery brought new insights on the bacterium as a pathogen, as well as on the relationship between genome size, genetic complexity and ecological versatility. This study unveiled that *P. aeruginosa* strain PAO1 had a 6.264 millions of base pairs genome, which is considerably larger than most of the bacterial genomes sequenced to date. *Pseudomonas aeruginosa* PAO1 genome encodes 5570 genes, of which more than 500 are involved in gene regulation, allowing the bacterium to switch on/off phenotypes required in specific ecological niches. Several other *P. aeruginosa* genomes were subsequently sequenced and comparative genomics has highlighted several features of

the *P. aeruginosa* pan-genome. Mathee et al. (2008) published the complete sequence and comparison analysis of five different *P. aeruginosa* strains. Five thousand and twenty one genes were conserved among all the five genomes, displaying at least 70% of sequence identity, and therefore they were considered to be part of the core genome. On the other hand, each genome carried up to 10% of unique sequences. Large inversions and recombination events were observed between strains, highlighting the high plasticity of *P. aeruginosa* chromosome (Figure 2). Moreover, at least 62 regions dispersed through the genome were associated with the presence of mobile genetic elements, suggesting that horizontal gene transfer play an important role in the evolution of *P. aeruginosa* genome (Figure 3).

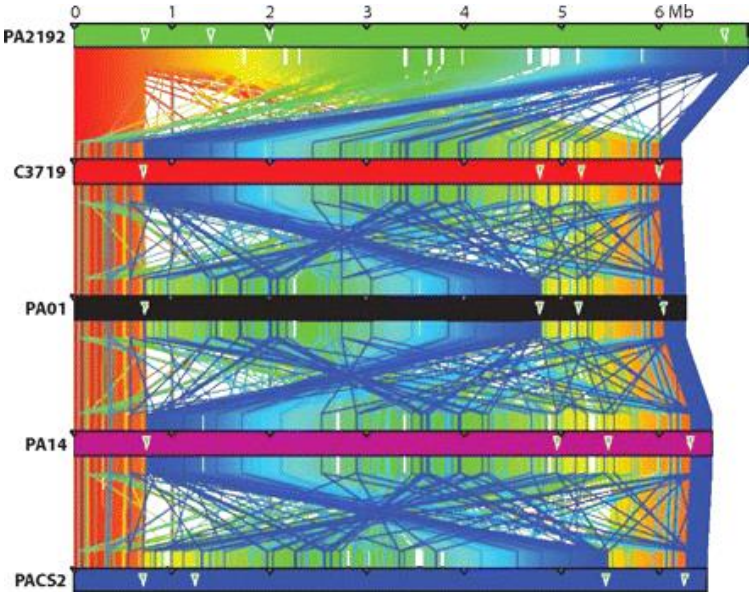


Figure 2. Whole genome alignments of five completely sequenced strains of *P. aeruginosa*. Representation of the large inversions in various *P. aeruginosa* chromosomes. At the top is seen the scale in Mb. Green, vertical arrowheads represent rRNA operons. PA2192 is given a color gradient so that corresponding alignment blocks may be easily visualized in the compared genomes (Mathee et al. 2008).

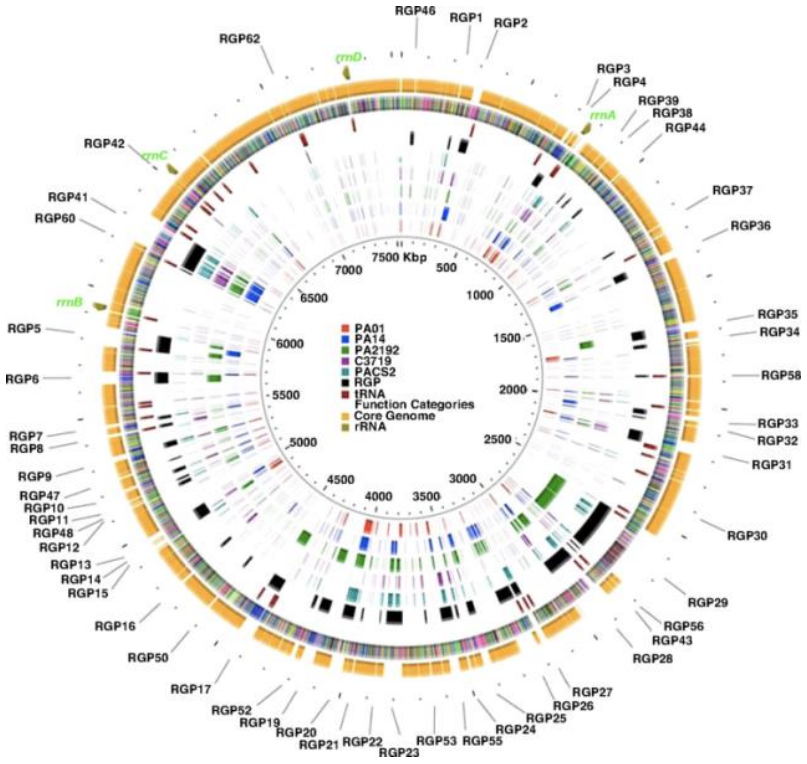


Figure 3. *Pseudomonas aeruginosa* pan-genome. The outer circle (gold) represents the core genome common to all strains, and the second circle the functional annotations. Third circle indicates tRNAs position. Gaps in the core genome represent the accessory genome, which is specific to each strain. Accessory genes in the inner circles are from PA14 (blue) PA2192 (green), C3719 (purple), PAO1 (red), and PACS2 (teal). Outer green arrows show the rRNAs positions (Mathee et al. 2008).

2.3. Pathogenicity of *P. aeruginosa*

2.3.1. Hosts and clinical outcomes of infection

Pseudomonas aeruginosa is an opportunistic pathogen that disseminates from its reservoirs and infects animals and humans, causing in the latter case infections in both community and hospital settings (Pirnay et al. 2009). Clinical outcomes of community-acquired *P. aeruginosa* infections can be, among others, ulcerative keratitis, external otitis, and skin and soft tissue infections (Driscoll et al. 2007). In hospitals, *P. aeruginosa* is responsible for more severe and invasive diseases in critically ill and immunocompromised patients (Sadikot et al. 2005). Notably, this bacterium is the main cause of nosocomial pneumonia in ventilated patients (Chastre and Fagon 2002).

Additionally, it can cause chronic airway infections in patients with bronchiestasis, chronic obstructive bronchopulmonary disease and cystic fibrosis (CF) (Cramer et al. 2012; Nicotra et al. 1995). Neutropenic patients undergoing chemotherapy often suffer from Bacteraemia caused by *P. aeruginosa*, as the case of patients with acute leukemia, in which 14-21% of Bacteraemia episodes are caused by this pathogen (Chatzinikolaou et al. 2000). Bacteraemias caused by this microorganism can also occur in immunocompromised HIV patients, in which the observed incidence rate was 10 times higher than general population (Driscoll et al. 2007). *Pseudomonas aeruginosa* is the third leading cause of nosocomial urinary tract infections (UTIs) (12 %), which occur through ascending and descending routes, and usually after catheterization or surgery (Hamasuna et al. 2004; Mesaros et al. 2007).

When the barrier function of the skin is compromised, as is the case of burned patients or patients with toxic epidermal necrolysis, it is extremely probable that an individual will be exposed to *P. aeruginosa* during the healing process, due to its high occurrence in the environment (Lyczak et al. 2000). Treatment of burned patients infected with this bacterium is extremely difficult, resulting in a mortality rate of 40-50 % (Estahbanati et al. 2002). Additionally, this pathogen is frequently isolated from patients immunocompromised by diabetes mellitus, for example, from diabetic foot infections (Driscoll et al. 2007).

2.3.2. Antimicrobial resistance

Pseudomonas aeruginosa infections have always been difficult to treat using antimicrobial chemotherapy (Mesaros et al. 2007). The problematic of *P. aeruginosa* resistant strains deserves special attention in many hospitals worldwide, since they are related with a three-fold higher rate of mortality, a nine-fold higher rate of secondary bacteraemia, a two-fold increase in the length of hospital stay, and consequently, a real burden in healthcare costs (Giamarellou 2002; Tumbarello et al. 2011). In 2000, the complete sequencing of the *P. aeruginosa* PAO1 wild strain, reported by Stover et al., enabled the gain of a great deal of information about this microorganism's inherent resistance. The total number of genes coding for proteins involved in antimicrobial

resistance mechanisms are present in a percentage of 0.3. In addition, due to the genome's flexibility, 10 % of the genes are organized in "pathogenicity islands" that possess variable genes coding for virulence factors, and capable to easily acquire large mobile genetic elements that codify for resistance genes (Stover et al. 2000). This pathogen's genome complexity and versatility is probably the basis of its capacity to resist a wide variety of antimicrobial agents, posing a serious problem in the choice of therapeutic strategies for serious infections (Hirsch and Tam 2010).

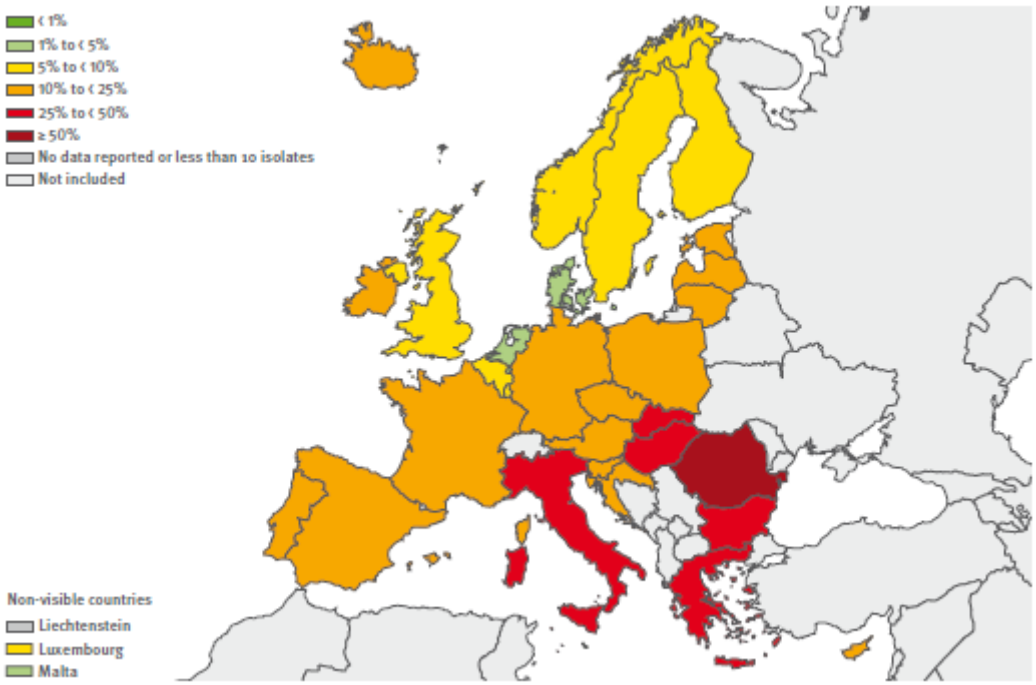
Pseudomonas aeruginosa possesses an intrinsic resistance to most antibiotics and wild type strains are inherently more resistant when compared to other Gram-negative bacterial species. The principal mechanisms leading to this form of resistance are its low outer membrane permeability, which is 12-100 times less than that of *E. coli*, the presence of multidrug efflux transporters, and endogenous antimicrobial inactivation (Breidenstein et al. 2011; Morita et al. 2014). Additionally, this bacterium can became even more resistant to antimicrobials due to acquisition of inheritable traits. Such mode of acquired resistance occurs via chromosomal mutations and horizontal transfer of genetic elements, like plasmids, transposons, integrons, prophages and resistance islands obtained by conjugation, transformation or transduction. Another type of resistance, which depends on the exposure of an antibiotic or environmental stimulus, is called adaptive resistance. When bacteria grow in the presence of a drug they may find ways to adapt to this new environment and acquire resistance to the antimicrobial agent in question (Skiada et al. 2011). This way of resistance comprises a number of triggering factors responsible for its induction, including antibiotics, biocides, polyamines, pH, anaerobiosis, cations and carbon sources, as well as mechanisms like biofilm formation and swarming (Fernandez et al. 2011).

The main classes of current anti-pseudomonal agents include β-lactams, fluoroquinolones and aminoglycosides (Mesaros et al. 2007; Morita et al. 2014; Poole 2011). Resistance to β-lactams, such as penicillins or carbapenems, is increasing and the resistance mechanisms involve β-lactamases, chromosomally encoded efflux mechanisms that lead to antibiotic expulsion, and a decrease of porins in the outer membrane, which

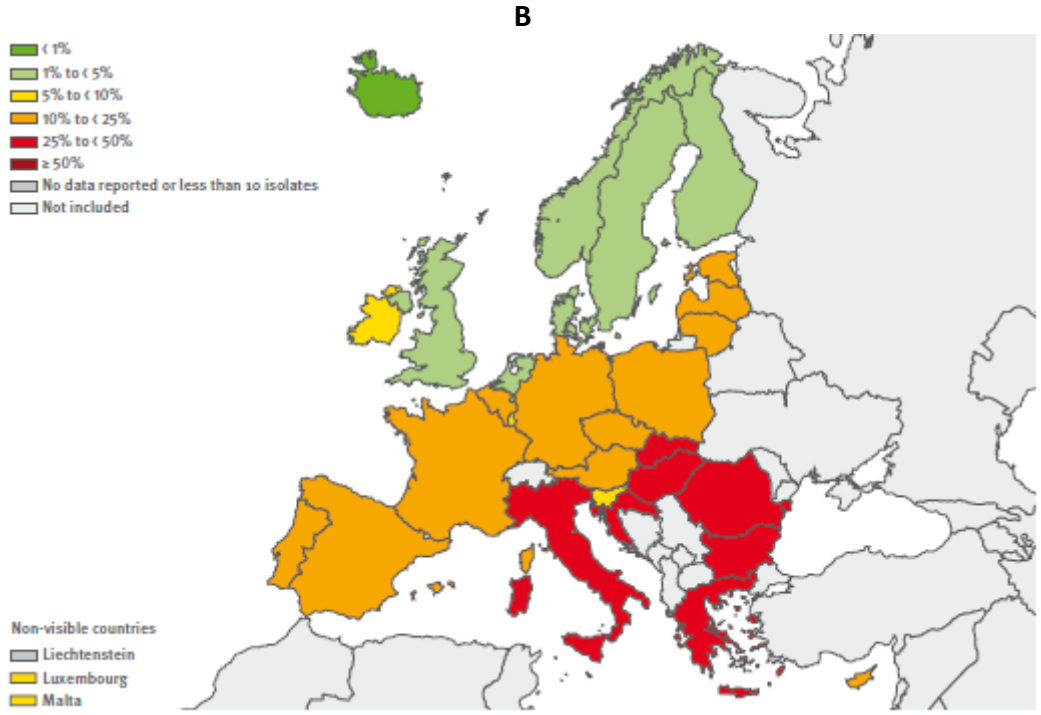
reduce the uptake of the drug (Pfeifer et al. 2010; Poole 2005; Zilberberg et al. 2010). Resistance to fluoroquinolones, ciprofloxacin in particular, involves mutations in the DNA gyrase and topoisomerase IV genes (Drlica et al. 2009; Jacoby 2005). Aminoglycosides, as amikacin and tobramycin, are used as treatment for patients with CF suffering pulmonary infections caused by *P. aeruginosa* (Taccetti et al. 2008). However, due to acquired aminoglycoside-modifying enzymes, rRNA methylases and endogenous efflux mechanisms, this class of anti-pseudomonal antibiotics is associated with high resistance occurrence (Poole 2005). Acquisition of plasmids that contain multiple resistance cassettes can lead to multi-resistant *P. aeruginosa* (MRPA) (Breidenstein et al. 2011). Similarly, the acquisition of other mobile genetic elements or the accumulation of multiple chromosomal changes over time can also lead to a multidrug resistance phenotype (Lister et al. 2009). An ICU surveillance study stated an increase from 13% to 21% in the prevalence of multidrug-resistant strains of *P. aeruginosa* from 1997 to 2002 (Livermore 2002). These prevalence percentages continued to augment until nowadays, being increasingly problematic in hospital settings, specifically in ICUs (Lister et al. 2009). Multi-resistant *P. aeruginosa* increased prevalence led to the return of polymyxin B and colistin, members of the antimicrobial cyclic oligopeptides family, which are now used as a last-resort treatment option, even if they cause strong side effects with high incidence (Poole 2011).

The European Antimicrobial Resistance Surveillance Network (EARS-Net) reported the percentages of *P. aeruginosa* isolates with resistance to carbapenems, aminoglycosides, fluoroquinolones, as well as multi-resistance occurrence for the year 2012 (Control 2013) (Figure 4). In general, high percentages of *P. aeruginosa* resistant isolates were observed. Most of the countries included in the surveillance showed percentages of resistance above 10% for all antimicrobial groups, despite the implementation of clinical guidelines to overcome this problem (Control 2013).

A


< 1%
1% to < 5%
5% to < 10%
10% to < 25%
25% to < 50%
≥ 50%
No data reported or less than 10 isolates
Not included
Non-visible countries
Liechtenstein
Luxembourg
Malta

B


< 1%
1% to < 5%
5% to < 10%
10% to < 25%
25% to < 50%
≥ 50%
No data reported or less than 10 isolates
Not included
Non-visible countries
Liechtenstein
Luxembourg
Malta

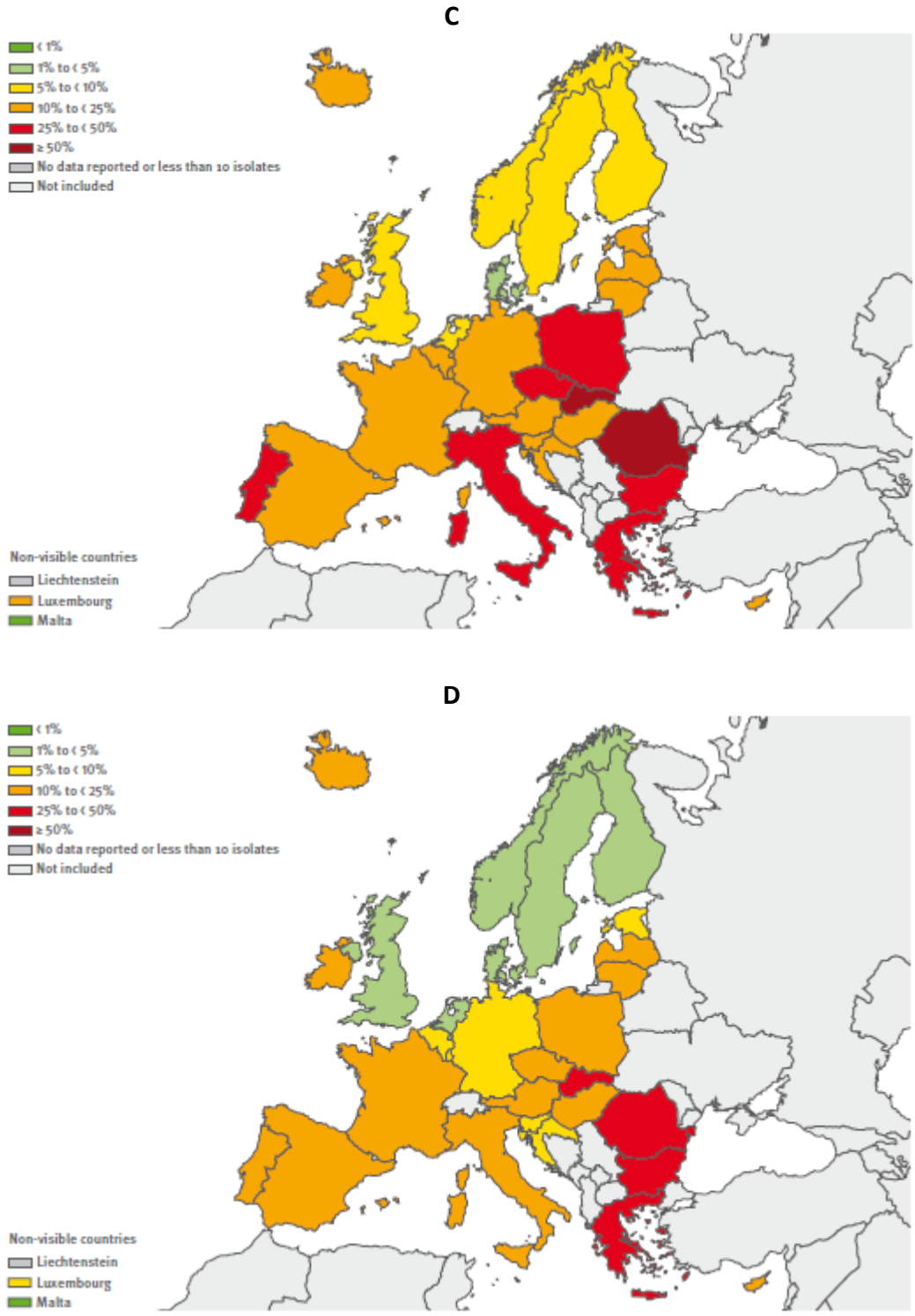


Figure 4. Percentage (%) of *P. aeruginosa* invasive isolates with resistance to several antimicrobial agents. Resistance to **(A)** carbapenems, **(B)** amynoglycosides, **(C)** fluoroquinolones, and with **(D)** multi-resistance to three classes of antimicrobial agents (β-lactams, fluoroquinolones and aminoglycosides) (Control 2013).

2.3.3. Virulence factors

Pseudomonas aeruginosa genome encodes many different virulence factors and regulatory mechanisms coordinating their expression, thus enabling the infection of several sites and persistence in hostile environments (Kipnis et al. 2006). Encouraged by the urgent need of new therapeutic agents that can successfully treat *P. aeruginosa* infections, technological advances in the fields of genomics, proteomics and microscopy are increasingly enlightening the pathogenicity of this bacterium (Veesenmeyer et al. 2009). The most common virulence factors are represented in Figure 5.

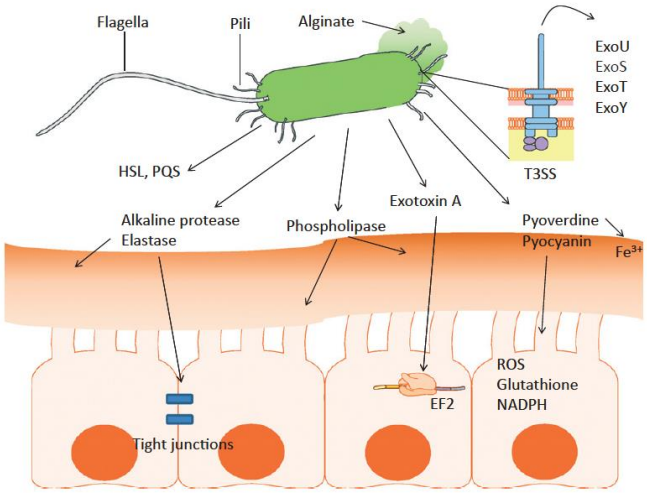


Figure 5. Several virulence factors associated with *Pseudomonas aeruginosa (Gellatly and Hancock 2013)*.

Many virulence determinants are associated with the bacterial surface. They include, among others, flagella, pili, lipopolysaccharides (LPS), components enabling toxin secretion, biofilm formation and quorum sensing (QS) (Gellatly and Hancock 2013; Kurahashi et al. 1999). *Pseudomonas aeruginosa* cell possess a single flagellum at the pole and shorter pili localized in the same region (Figure 5). The flagellum provides swimming motility in aqueous environments, but it is also very important in biofilm dispersal and adhesion to host cells (O'Toole and Kolter 1998; Shan et al. 2008). Pathogenicity mechanism of this virulence factor consists in the recognition of flagellin (structural unit of the flagellum) by toll-like receptor 5 present in the host cell surface. Subsequently, this

event triggers an immune response as a consequence of cytokines synthesis (McIsaac et al. 2012). Non-flagellated mutants in models of acute infection suffer a virulence loss, which supports even further the importance of this structure in *P. aeruginosa* pathogenicity (Feldman et al. 1998). Multiple pili or fimbriae are also present on the *P. aeruginosa* surface (Figure 5). Among them, type IV pili are important adhesins enabling the "twitching motility", consisting of retractile movements that pull bacteria along solid surfaces (Mattick 2002). Pili can also lead to bacterial aggregation forming microcolonies in specific tissues, providing protection from immune system responses and antimicrobial treatment (Sriramulu et al. 2005).

LPS are structures located on the outer membrane, whose detection generates a strong immune response, triggering the inflammatory response, exclusion of external molecules, and enabling interactions with antimicrobial agents (King et al. 2009; Kipnis et al. 2006). LPS is a three-domain complex glycolipid consisting of a Lipid A, a polysaccharide core region and a highly variable O-specific polysaccharide. Lipid A has the role of anchoring the lipopolysaccharide into the outer membrane and it can activate a series of pro-inflammatory cascades (Wieland et al. 2002). Antigenic identification of *P. aeruginosa* serotypes is mostly due to variable O-specific polysaccharide chains, which makes this molecules real targets for immunotherapy (Hatano and Pier 1998)

Quorum sensing and the ability to form biofilms further increase the pathogenic power of *P. aeruginosa*. Quorum sensing infers about local bacterial population density and regulates the gene expression accordingly (Figure 5). About 350 genes (6% of the *P. aeruginosa* genome) are regulated by QS systems, which also play an important role in biofilm formation and toxin production (Deep et al. 2011; Schuster et al. 2003). Several studies using QS mutants defective for the main genes responsible for this phenomenon showed that, if the bacterium is not able to perform QS, its pathogenicity is compromised (Rumbaugh et al. 1999; Sandoz et al. 2007; Wu et al. 2001). Biofilms are highly organized and structured microbial communities, encased in extracellular polymeric substances (EPS) and attached to the surface. EPS are polymers of polysaccharides, nucleic acids, lipids and proteins that make up to 90 % of the biofilm volume and confer physical and chemical robustness to the structure (Bjarnsholt et al. 2010; Lieleg et al. 2011) Biofilm

formation enables the growth of bacteria in a protective mode, allowing them to survive in hostile environments (Wei and Ma 2013).

Toxin secretion also plays an important role in the virulence of *P. aeruginosa*. Type 3 secretion system (T3SS) is a complex pilus-like structure that enables the translocation of effector proteins from bacteria, through bacterial membranes and into the host cytoplasm using a needle-like appendage that forms a pore in the eukaryotic membrane (Sawa and Wiener-Kronish 2004). They are also present in other pathogenic genera, like *Yersinia*, *Salmonella* or *Shigella* (Coburn et al. 2007). In *P. aeruginosa*, only four effectors have been characterized until now: ExoY, ExoS, ExoT, and ExoU (Le Berre et al. 2011). The expression of this secretion system is usually associated with acute invasive infections, frequently leading to high mortality rates (Hauser 2009).

Alginate is a copoplymer of beta-D-mannuronic acid linked by 1-4 linkages to an alpha-L-guluronic acid (May et al. 1991). Secretion of this exopolysaccharide at the cell surface enhances the adhesion capacity and anchor *P. aeruginosa* to the colonized respiratory epithelium, as in the case of respiratory infections (Davies 2002). In addition, other secreted proteins may play an important role in *P. aeruginosa* virulence, as it is the case of pyocyanin, pyoverdine, alkaline protease, protease IV, elastase, phospholipase and exotoxin A (Kipnis et al. 2006).

3. Definition of isolate, strain and clone

Before approaching microbial typing it is of great importance to define the three most vastly used terms in this field, i.e. ”isolate”, “strain” and “clone”. A bacterial isolate can be defined as a population of bacterial cells in pure culture derived from a single colony. In clinical microbiology, isolates are normally derived from a primary culture of a clinical specimen recovered from a patient. On the other hand, a bacterial strain consists of an isolate or a group of isolates that are indistinguishable by phenotypic and/or genotypic characteristics. Ultimately, two isolates can represent one strain, but the contrary does not apply (van Belkum et al. 2007). The term “clone” is frequently used in the context of bacterial epidemiology and population genetics, and is defined as bacterial cultures isolated from different sources in different locations, possibly at different periods

of time, which still possess so many identical phenotypic and genotypic characteristics that this relation could be explained by they being derived from a common ancestor, within a relevant life span (Dijkshoorn et al. 2000).

4. Typing of microorganisms

The main role of bacterial typing is to unravel clonal relatedness between different isolates within a species (Sabat et al. 2013). This enables the assessment of the sources and routes of infection, confirms or rules out outbreaks, determines cross-transmission of nosocomial pathogens, recognizes virulent strains and evaluates the effectiveness of the control measures being applied, as well as of the surveillance systems (MacCannell 2013; Perez-Losada et al. 2013). Typing of microorganisms rely on the fact that bacterial genomes are continuously shaped by genetic mechanisms including point mutations, recombination, gene loss or acquisition and horizontal gene transfer (Francino 2012; Lawrence 1999). This genetic diversity within bacterial species results in the creation of new phenotypes, which may have selective advantages in specific ecological niches (Cordero and Polz 2014).

There are two different typing systems currently in use, one based on the microorganism phenotype (Table 1), and the other on its genotype (Table 2). Nevertheless, the analyses of bacterial phenotypic characteristics show lower discriminatory power than genotypic characters (Foxman et al. 2005; Li et al. 2009). Genotyping techniques currently used can be divided in three main groups: methods based on DNA banding patterns, based on DNA sequencing and based in DNA hybridization (Foxman et al. 2005; Li et al. 2009; Ranjbar et al. 2014) (Table 2).

Several considerations need to be taken into account when choosing a molecular typing method, and they greatly depend on the need of resolution, on the epidemiological context, as well as on the time and geographical scale it is going to be applied (Sabat et al. 2013). The different characteristics desirable for a molecular technique are present in Table 3. When choosing the ideal genotyping method, the presence of specific characteristics is crucial. Regarding the performance of the method, it should have intra- and inter-laboratory reproducibility, interlaboratory portability, and

unequivocal interpretation of results, high throughput and appropriateness. In terms of convenience, it must be user-friendly, with low cost, rapid and affordable (Gurtler and Mayall 2001; van Belkum 2003). However, between all the molecular typing methods available, none can be consider universally ideal, since they all got their strengths and weaknesses (Li et al. 2009)

Table 1. Description of the phenotypic methods currently used for bacterial typing.

Typing technique	Principle	Advantages	Disadvantages
Biotyping	Analysis of colony morphology; biochemical tests	• Easy to perform • Automated • Coupled to identification systems (API, e.g.)	• Low discriminatory power • Low reproducibility (can be affected by technical and environmental factors)
Serotyping	Detection of bacterial determinants	• Easy and fast • Automated for some bacteria	• Expensive or non commercialized antisera • Not all strains are serotypable • Low discriminatory power • Instability of antigenic determinants due to recombination
Phage typing	Sensibility to a series of phages (resistance or lysis)	• Subdivision of serotypes • Typing of nonserotypable strains	• Intricate equipment • Difficulty of active phages and control strains availability • Lack of standardization • Low reproducibility
Antibiogram	Sensibility to different antibiotics	• Easy to perform • Automated • Allows the identification of multi-resistant phenotypes	• Low discriminatory power • Genetic variability of antibiotic resistance • Selection pressure

4.1. Molecular typing of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa possesses a very complex ecology and only powerful typing methods can give insight on the relatedness of strains, and consequently on the routes of colonization and/or infection (Blanc et al. 2004). This pathogen's epidemiology has been analyzed by a series of different molecular typing methods.

The high discriminatory power of Pulsed-field gel electrophoresis (PFGE) makes it one of the most powerful molecular methods currently used, often referred as the "gold standard" for DNA fingerprinting of many microorganisms, as in the case of *P. aeruginosa* (Gautom 1997; Salimi 2009; Tenover et al. 1995). However, this method possesses several inconvenients, like long analysis time, the use of expensive and specialized equipment, low intra- and inter-laboratory reproducibility and is labor-intensive, which make it not the optimal method to be used in a large investigation (Botes et al. 2003; Tenover et al. 1995). Multiple-locus variable number tandem repeat (MLVA), which characterizes each isolate by the number of repeats in several loci, is another example of a molecular typing technique that has been applied in different *P. aeruginosa* typing schemes (Sobral et al. 2012; Vu-Thien et al. 2007). Nevertheless, the definition of types is ambiguous hindering inter-laboratory standardization (Li et al. 2009). Multi-locus sequence typing (MLST) relies on partial sequencing of seven genes of the core genome and showed to be efficient in the study of the global population structure of *P. aeruginosa* (Wiehlmann et al. 2007). Several studies on *P. aeruginosa* population genetics were performed using this technique, but it was not discriminatory enough to investigate local epidemiology (Vernez et al. 2005).

In the past decade, DNA sequencing technologies have made important improvements, both quantitatively and qualitatively, increasing accessibility of this technology to research and clinical laboratories worldwide. After the completion of the first human genome sequence (Human Genome Sequencing 2004), different projects aiming to create new cheaper and faster sequencing methods resulted in the development of next generation sequencing (NGS) methods (Grada and Weinbrecht 2013). Whole genome sequencing (WGS) enables a single base-pair resolution between isolates, making it an ultimate molecular typing technique to study bacteria. Several

recent studies on *P. aeruginosa* evolution and dissemination in hospital settings have been performed (Dettman et al. 2013; Jelsbak et al. 2007; Snyder et al. 2013). Unfortunately, the use of this method in diagnostic laboratories is still very expensive, and the analysis of results is complex, time consuming, and not yet standardized (Caulfield et al. 2013; Meynert et al. 2013; van El et al. 2013).

4.1.1. Double locus sequence typing (DLST)

Double locus sequence typing is a DNA sequence-based method that relies on partial sequencing of two highly variable loci, and it has been successfully used to investigate the epidemiology of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Basset et al. 2009; Basset et al. 2010; Basset and Blanc 2014; Kuhn et al. 2007; Sakwinska et al. 2010). Similarly to other sequence-based methods, it gives unambiguous definition of types, allowing inter-laboratory comparisons and high reproducibility. In addition, the use of 96-well microtiter plates greatly reduces costs and handling time. For such reasons, this method can be incorporated into long term routine surveillance programs (Basset et al. 2009; Basset et al. 2010). In the case of *P. aeruginosa*, the two hypervariable loci consist in *ms172* (partial sequencing of 400 base pairs), and *ms217* (350 base pairs) (Basset and Blanc 2014). A simple representation of both loci is present in Figure 6. For both loci, an arbitrary number is assigned to each allele that has a distinct sequence. Hence, the final result consists in two numbers that correspond to the DLST type, for each isolate (Basset 2014).

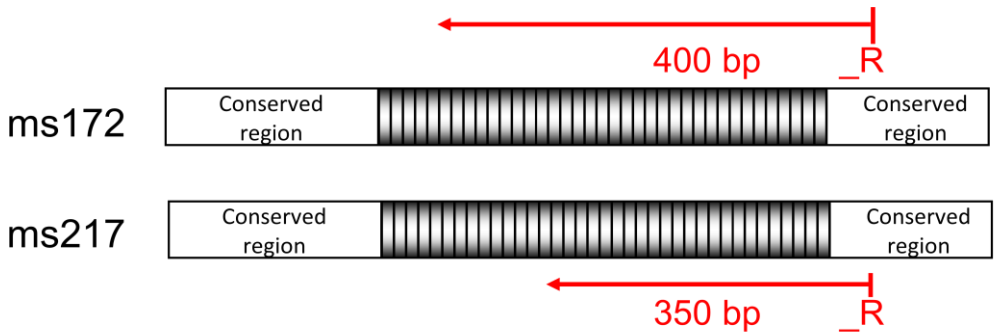


Figure 6. Hypervariable loci *ms172* (400 base pairs) and *ms217* (350 base pairs) used in the typing of *P. aeruginosa* with DLST.

Table 2. Description of the most common genotypic methods for bacterial typing (Foxman et al. 2005; Li et al. 2009; Ranjbar et al. 2014).

	Typing technique	Principle	Advantages	Disadvantages
DNA banding-pattern-based methods	**PFGE**	Digestion of DNA with rare cutting-endonucleases, generating large DNA fragments (10 kb to 10 Mb). By applying alternating electric fields, at different angles, restriction fragments can be separated in an agarose gel	• High discriminatory power • High epidemiological concordance • Relatively inexpensive • High typeability	• Labour intensive • Specialized personnel to interpret the results • Poor portability • Technical problems (weak intensity of banding patterns, artifactual bands, and asymmetric lanes)
	RFLP	Digestion of DNA with frequently cutting-endonucleases. Fragments are separated by a conventional agarose gel electrophoresis, and transferred to a membrane via Southern blotting, with labeled probes	• Relatively cheap equipment • Cost effective	• Requires large amounts of high-quality genomic DNA • Time and labor consuming • Makes use of radioisotopes and complex biochemistry
	RAPD-PCR	Random amplification of unknown genomic regions using arbitrary primers. Makes use of short single primers, coupled with low annealing temperatures. Genomic DNA is amplified at multiple loci, generating different-sized amplicons, which are separated by agarose gel electrophoresis	• Do not require knowledge of specific DNA target sequences (general applicability) • Short turn-around time • Inexpensive and sensitive	• Low inter- and intra-laboratory reproducibility • Lack of consensus rules to interpret results

	Typing technique	Principle	Advantages	Disadvantages
	REP-PCR	Primers complementary to interspersed repetitive consensus sequences are used to amplify DNA fragments between repetitive elements. Amplicons are then separated by agarose gel electrophoresis or capillary electrophoresis	• Low cost of materials • Low labor intensity • Fast and easy • Semi-automated commercial kit: DiversiLab System	• Low inter-laboratory reproducibility • Potential for contamination • Occurrence of artifacts • Need for multiple controls
	MLVA	Polymorphic analysis of multiple variable tandem repeats (VNTR) loci on the chromosome. For each VNTR locus, the number of repeats can be determined by PCR. PCR amplicons are fluorescent labeled and separated by capillary electrophoresis	• High discriminatory power • Rapid, easy to perform and inexpensive • Reproducible	• Unsuitable for long-term epidemiological surveillance • VNTR loci are not always common in bacterial genomes • Size differences may not always reflect the real number of tandem repeats
DNA sequence -based methods	**Pyrosequencing**	Non-electrophoretic sequencing method based on real-time quantitative detection of pyrophosphate release following nucleotide incorporation into a DNA chain during its assembly	• High-throughput • Generates DNA sequences rapidly • Potential for scale-up	• Limited by short-read lengths (25-250 bp) • Difficulty of sequence assembly
	MLST	Fragments of 450-500 bp of seven housekeeping genes are sequenced. For each locus, an arbitrary number is assign for each distinct allele	• Portable • High inter-laboratory reproducibility • Makes use of housekeeping genes, being useful to understand global population structure	• Expensive • Time consuming • Discriminatory power too low to investigate local epidemiology

	Typing technique	Principle	Advantages	Disadvantages
	WGS	Sequencing of the entire genome	• High discriminatory power • High repeatability • High reproducibility	• Very expensive • Time consuming (analysis) • Subjective interpretation of results
DNA hybridization-based methods	**Macroarrays**	Hybridization of approximately 5000 DNA fragments or oligonucleotides (spots) arrayed on a substrate (membranes, glass, plastic, silicon wafers, or metal alloys)	• Rapid • Specific • Cost-efficient	• Lower discriminatory power than microarrays
	Microarrays	Same principle as Macroarrays, but much denser, with up to 1 000 000 spots per array	• High-throughput • High discriminatory power	• Indirect measure of relative concentration • Linkage of related DNA/RNA sequences to the same probe • Need previous knowledge about the sequence

4.1.2. Double digest selective label (DDSL)

Another molecular typing method called Double Digest Selective Label (DDSL) has been used to type *P. aeruginosa* isolates (Terletskiy et al. 2008). The principle of this method is very similar to the one of PFGE, but instead of separating large chromosomal fragments, it relies on the migration of smaller segments of chromosomal DNA. Genomic DNA is subjected to digestion by two restriction enzymes, one that cuts rarely (*Spe*I) and a second that cuts frequently (*Stu*I). This results in digestion of DNA fragments that are easily separated in a regular agarose gel (Terletskiy et al. 2008). In parallel to digestion, DNA fragments containing *Spe*I-digested ends are labeled by biotin and they are revealed once the separated fragments have been blotted to a membrane, giving a specific fingerprinting pattern.

In the same investigation performed by Basset and Blanc previously mentioned (Basset and Blanc 2014), DDSL was used as the reference when compared with DLST. The results for DLST typing were in general very similar to those of DDSL. However, the latest showed a higher discriminatory power, making it a putative ideal typing complement to resolve DLST clusters in specific situations (Basset and Blanc 2014).

5. Epidemiology of *Pseudomonas aeruginosa* in ICUs

Pseudomonas aeruginosa accounts for 11 – 14% of nosocomial infections. These values reach even higher percentages, 13 – 23%, when the infection is acquired in ICUs (Erbay et al. 2003; Gaynes et al. 2005). Upon admission in ICUs, approximately 2 – 13% of individuals are colonized with *P. aeruginosa*, and 1% is infected with this pathogen (Bertrand et al. 2001). A study performed in the USA reported that *P. aeruginosa* was considered the third most frequent microorganism causing wound and pulmonary infections, the fourth most frequent microorganism causing UTIs, and the fifth most frequent microorganism isolated from patients in septicemia (Richards 2000). Although its incidence may vary from unit to unit, and from study to study, *P. aeruginosa* is commonly identified as the most frequent microorganism in burn units, being the cause of a large number of wound infections, bacteraemia and ventilator-associated pneumonia in these units (Lari and Alaghehbandan 2000; Yildirim et al. 2005). Additionally, despite

Gram-positive bacteria being a more common cause of nosocomial bloodstream infections, *P. aeruginosa* has been associated with higher mortality rates (Osmon et al. 2004).

A general overview of the *P. aeruginosa* epidemiology in the ICU suggests that colonization is a crucial aspect to be taken into account, since it represents the true bacterial load within ICUs (Bertrand et al. 2001; Wunderink and Mendoza 2007). Outbreak occurrence in ICUs was thought to be mainly caused by environmental sources. Thus, after implementation of control measures, several studies showed a reduction of outbreaks (Cobben et al. 1996; Lanini et al. 2011). In addition to environmental reservoirs, *P. aeruginosa* was also found to be part of the endogenous microbiota of 2.6 to 24% of the hospitalized patients (Blanc et al. 2007; Morrison and Wenzel 1984). Currently, the endogenous microbiota is thought to be the origin of most nosocomial infections. Colonized/infected patients can act as exogenous sources of infection and be responsible for the infection of successive hospitalized patients (Bertrand et al. 2001). In order to track the dissemination of this bacterium it is very important to determine the source of infection. To do so, an extensive screening of the environment and a study of the patients' endogenous microbiota are crucial, but also require significant resources (Cuttelod et al. 2011).

Table 3. Different characteristics of a molecular typing technique (Sabat et al. 2013).

Characteristic	Definition
Typeability	Ability to generate a result for each isolate
Repeatability	Ability to generate identical results when an isolate is tested several times in the same laboratory
Reproducibility	Ability to generate identical results when an isolate is tested several times in different laboratories
Discriminatory power	Ability to generate distinct information from epidemiologically unrelated isolates
Stability	Ability to determine a clonal relationship between isolates in spite of differences accumulating during their spread in time or space
Ease of data interpretation	Unequivocal interpretation
Ease of use	Ability to be used in non-specialized and non research laboratories, by workers with minimal training
High throughput	Ability to process a large number of strains in a relatively short interval of time
Cost and affordability	Depends on the cost and accessibility of reagents and equipment, as well as the skills required
Typing system concordance	Ability to determine strains epidemiological relatedness when the typing method is tested against evidence obtained with a method already validated
Epidemiological concordance	Ability to determine strains epidemiological relatedness when the typing method is tested against epidemiological evidence
Phylogenetic information	Ability to give information on isolates evolutionary relationship
Appropriateness	Ability to address the epidemiological investigation in question

5.1. Lausanne University Hospital setting

In 1998, a molecular epidemiological investigation on *P. aeruginosa* possible sources and transmission was performed in the University Hospital of Lausanne (Blanc et al. 2004). It reported that the acquisition of *P. aeruginosa* from faucets as an exogenous source was an important cause of infection and colonization in ICU patients, during a non-epidemic period. Infection control measures were implemented and consequently decreased the incidence of *P. aeruginosa* infection and colonization in patients hospitalized in the ICU, showing its efficiency in the presence of an environmental reservoir (Petignat et al. 2006).

A more recent study conducted in the same hospital investigated whether *P. aeruginosa* infections in ICU patients were due to endogenous or exogenous sources (Cuttelod et al. 2011). This study covered a period of 10 years, from 1998 to 2007, and recurred to the molecular typing techniques PFGE (1998-2004) and DDSL (2007). The authors concluded that the relative contribution of endogenous and exogenous reservoirs to the colonization and infection of ICU patients varies over time. Such variations are most likely influenced by the environment contamination, the following of infection control measures by healthcare workers, and by *P. aeruginosa* strains.

II. Objectives

Double locus sequence typing has been successfully used as a typing tool for epidemiological investigations at the University Hospital of Lausanne, Switzerland. However, in some circumstances, this method was not discriminatory enough to resolve clusters of related strains that belonged to the same DLST type. DDSL has been proven to have a higher discriminatory power compared to DLST for typing *P. aeruginosa* isolates (Basset and Blanc 2014). Therefore, the main objective of this study was to use DDSL in order to decipher DLST clusters and achieve a better concordance between genetic and epidemiological clusters. To make it possible, the following tasks were conducted:

- Technical optimization of the DDSL typing method to acquire results with better quality for analysis;
- Typing *P. aeruginosa* isolates correspondent to 4 of the genetic clusters determined by DLST;
- Gather and analyze epidemiological data of all patients sharing the same DLST genetic cluster;
- Compare both the molecular and epidemiological information.

III. Material and Methods

1. Setting

The University Hospital of Lausanne is an 1100-bed tertiary healthcare setting, which comprises an adult Intensive Care Unit (ICU), including a burn unit, with about 1910 admissions per year.

2. Bacterial isolates, DLST typing and epidemiological data

Between January 2010 and July 2012, *P. aeruginosa* was recovered from clinical specimens in 272 patients that were hospitalized in the ICUs. From these, 689 isolates were obtained from 254 patients (93.4%). All the isolates were previously typed by the double locus sequence typing (DLST) method. Patients that shared the same DLST genotype were defined as belonging to the same "genetic cluster". A total of 46 clusters were identified. The four major clusters where DLST 1_18 (25 patients, 82 isolates), DLST 1_21 (15 patients, 24 isolates), DLST 6_7 (14 patients, 31 isolates) and DLST 28_77 (12 patients, 15 isolates). Isolates (N=152) from these clusters were further analyzed in this study.

Epidemiological data (unit and room of hospitalization, dates of ICU admission and discharge, age, sex, clinical diagnosis and death occurrence) was retrieved from the hospital databases and used to construct epidemiological maps. Epidemiological links between patients or environment were identified as: (i) patients hospitalized during overlapping periods in the same ICU, or (ii) patients showing an identical DLST type with an environmental sample isolated in the same room during the period of the study.

3. Genomic DNA extraction methods for DDSL analysis

Extraction of DNA was performed with three commercially available kits, in order to find an extraction method yielding high quality genomic DNA suitable for DDSL analysis (see below). DNA quantity and quality was monitored using ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington DE, USA).

3.1. QuickGene DNA tissue kit S[1]

Pseudomonas aeruginosa isolates were grown overnight on blood agar plates at 37 °C and bacterial biomass from approximately half culture plate was suspended in 200 µL of 0.9% NaCl solution. The bacterial suspension was then transferred to lysing matrix B tubes (MP Biomedicals, Solon, Ohio) containing 0.1 mm silica beads, and 500 µL of Lysis buffer mix, consisting of 480 µL of Lysis Buffer (LDT) and 20 µL of proteinase K (EDT), were added. Bacterial lysis was performed in the FastPrep-24 Instrument (MP Biomedicals) homogenizer using two cycles of 40 sec at 6.0 m/s. Samples were then heated at 70° C for 10 minutes and centrifuged at 14'000 rpm for 10 minutes. Supernatants were collected in new tubes and 450 µL of 99% ethanol were added to them. The samples were then transferred to QuickGene-Mini 80 system cartridges and first pressurization was applied, until all the liquid passed through the cartridge. Three washing cycles with 750 µL of Wash buffer (WDT) and pressurization were posteriorly performed. In the last step, the cartridge rack was moved to the elution position and 150 µL of Elution buffer (CDT) were added to each cartridge. After the final pressurization step, the DNA solution was obtained and subsequently stored at 4 °C for immediate use, or at -20 °C for an extended period.

3.2. Wizard Genomic DNA Purification Kit[2]

Single colonies were inoculated in 5 ml of LB and 1.5 mL of the resulting overnight cultures were centrifuged at 14'000 rpm for 2 minutes. Bacteria were then resuspended in 600 µL of Nuclei Lysis Solution and incubated at 80 °C for 15 minutes. Three microliters of RNase solution (4 mg/mL) were added to the cell lysates, followed by incubation at 37 °C for 60 minutes. After the addition of 200 µL of Protein Precipitation Solution, samples were vortexed and incubated on ice for 5 minutes. Samples were then centrifuged at 14'000 rpm for 3 minutes, and supernatants were transferred to new tubes containing 600 µL of isopropanol, and gently mixed. Followed a centrifugation step at 14'000 rpm for 2 minutes, DNA was washed twice with 600 µL of 70% ethanol. The DNA pellet was air-dried and dissolved in 100 µL of nuclease-free water (Gibco, Life Technologies, Zug,

[1] Kurabo/Fuji; biostep GmbH, Jahnsdorf, Germany
[2] Promega Corporation, Madison WI, USA.

Switzerland) by incubation at 65°C for 1 hour. DNA samples were stored at 4 °C, or at -20 °C for an extended period.

3.3. GenElute bacterial genomic DNA Kit[3]

Single colonies were inoculated in 5ml of LB and 1.5 mL of the overnight cultures were centrifuged at 14'000 rpm for 2 minutes. Bacterial pellets were resuspended in 180 µL of Lysis Solution T, supplemented with 20 µL of RNase A solution, and incubated at room temperature for 2 minutes. Twenty microliters of Proteinase K solution (20 mg/mL) were added to the samples, followed by an incubation of 30 minutes at 55 °C. Two hundred microliters of Lysis Solution C were added to the suspensions and incubated again at 55 °C, for 10 minutes. After the incubation period, 200 µL of 99% ethanol were added to the lysate and this mixture was thoroughly vortexed. GenElute Miniprep Binding Columns were washed with 500 µL of Column Preparation Solution. Cell lysates were transferred to the binding column, using wide-bore pipette tips to avoid DNA shearing and centrifuged at 14'000 rpm for 1 minute. The columns were washed with 500 µL of Wash Solution 1 and centrifuged at 14'000 rpm for 1 minute. A second washing step was performed using 500 µL of Wash Solution diluted with 99% ethanol. Columns were then dried by centrifugation at 14'000 rpm for 3 minutes. Nuclease-free water was heated at 60 °C and 100 µL were added in the column and incubated for 5 minutes at room temperature. DNA was eluted by centrifugation at 14'000 rpm for 1 minute. DNA solutions were stored at 4 °C for immediate use, and at -20 °C for longer-term storage.

4. DDSL fingerprinting protocol

4.1. Preparation of the biotinylated reference marker

Four micrograms of Lambda phage DNA (New England BioLabs, GmbH, Frankfurt, Germany) were digested with 1.6 U/µL of *Sty*I–HF endonuclease (New England BioLabs) in 1X CutSmart Buffer (New England BioLabs). The digestion mixture was incubated at 37 °C for 60 minutes and *Sty*I-HF was subsequently inactivated at 65 °C for 20 minutes. Biotinylation was performed as shown in Table 4, at 72 °C, for 3 minutes.

[3] SIGMA-ALDRICH, St. Louis, MO, USA.

Table 4. Mix used for the biotinylation of the reference marker.

	Amount	Final concentration
Lambda phage digested DNA	10.0 µL	40 ng/µL
Bio-14-dCTP	2.0 µL	0.04 mM
10X PCR Buffer	1.5 µL	0.75 X
$MgCl_2$	0.6 µL	1.5 mM
Taq DNA Polymerase	2.0 µL	0.5 U/µL
Nuclease-free H_2O	3.9 µL	
Total	20.0 µL	

4.2. Digestion and biotinylation of *P. aeruginosa* genomic DNA

For each isolate, double digestion/labelling mix was prepared as listed in Table 5, and genomic DNA was added. This mixture was then incubated at 37 °C for 2 hours**.**

Table 5. Mix used for the double digestion/labelling of *P. aeruginosa* genomic DNA.

	Amount	Final concentration
Genomic DNA	2.0 µL	20-30 ng/µL
Bio-14-dCTP	0.04 µL	0.8 µM
*Spe*I	0.4 µL	0.2 U/µL
*Stu*I	0.4 µL	0.2 U/µL
10X CutSmart Buffer	2.0 µL	1 X
100X BSA	0.2 µL	1 X
Taq DNA Polymerase	0.16 µL	0.04 U/µL
Nuclease-free H_2O	14.8 µL	
Total	20.0 µL	

After incubation, samples were desalted using refillable microcolumns containing 800 µL of Sephadex G-50 (GE Healthcare, Buckinghamshire, UK) previously prepared by centrifugation at 3'000 rpm for 2 minutes. A volume of 20 µL of digested DNA of each isolate was added to the center of the column containing Sephadex G-50, followed by centrifugation at 3'000 rpm for 2 minutes.

4.3. DNA fragments separation by electrophoresis

Three microliters of 6X DNA Loading Dye (Thermo Scientific) were added to 20 µL of each purified sample. DNA fragments were separated in a 20 cm 0.8% agarose gel (Agarose NA; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at 150 V for 3.5 hours in a 1X TAE buffer. The electrophoresis was carried out at 30 °C.

4.4. Transfer of the separated DNA fragments to a neutral nylon membrane

Immediately after the electrophoresis, separated DNA fragments were transferred to a neutral nylon membrane (Amersham Hybond-N; GE Healthcare). The transfer was performed during 40 minutes in a blotting system (Millipore AG, Zug, Switzerland) at 40 mbar, using deionized water. When the membrane was dried, transferred fragments were linked to the membrane by UV irradiation at 150 mJ/cm^2 in the GS Gene linker chamber (GS Gene Linker UV Chamber; Bio-Rad).

4.5. Membrane revelation

Prior to revelation, neutral nylon membranes containing the DNA fragments were washed during 1 minute with Buffer 1 (11.6 g maleic acid + 9.0 g NaCl + 8.05 g NaOH + 1L demineralized H_2O). Immediately after, the membranes were washed for 20 minutes with Buffer 2 (450 mL Buffer 1 + 45 mL 10x blocking solution (10 g Blocking Reagent powder (Roche) + 90 ml Buffer 1)). Membranes were then incubated at room temperature for 30 minutes with the conjugate solution containing 5 mL of Buffer 2 supplemented with 5 µL of Streptavidin-Alkaline Phosphatase (Bio-Rad). After two washing steps with Buffer 2 for 10 minutes, the developing solution containing the chromogenic substrates NBT and BCIP (Roche) was used to reveal the bands. This step was performed in the dark, at room temperature with mild agitation during 20 minutes. Membranes were then washed with deionized H_2O and dried completely.

4.6. Analysis of DDSL patterns

Membranes with DNA fingerprinting patterns were scanned in order to produce TIFF files with a 300 dpi resolution that were processed and analysed using BioNumerics 7.0

software (Applied Maths NV, Sint-Martens-Latem, Belgium). First, DDSL band profiles were compared to identify band differences between them, using the auto-assignment of bands option from this software. Cluster analysis was also performed recurring to curve based coefficients. In this case, similarity values between banding patterns were calculated with the Pearson's similarity coefficient based upon densitometry curves. In such curves a peak was drawn for each band, and the area under the peak was proportional to the intensity of the band, as shown in Figure 7.

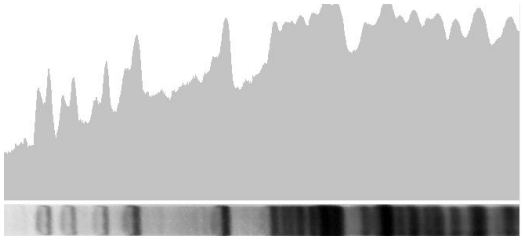


Figure 7. Example of a densitometry curve profile assigned by the BioNumerics software.

Similarity values were used in the construction of a similarity matrix, which in turn was converted into a dendrogram with a clustering algorithm. Additionally, a visual comparison of the different profiles was performed in order to manually determine the different DDSL types.

IV. Results

1. Optimization of the DDSL typing method

In order to increase the quality and the reproducibility of the previously described DDSL method (Terletskiy et al. 2008), several modifications were applied to the existing protocol.

1.1. Genomic DNA extraction method

The DNA extraction was found to be a crucial step of the procedure to obtain accurate and reproducible results. The published DNA extraction method of the DDSL protocol (Terletskiy et al. 2008) was based on the traditional single step phenol-chloroform extraction, which was time consuming, required hazardous agents and gave results with high inter-samples variability (data not shown). To standardize and simplify this step, we tested three commercially available DNA extraction kits on seven *P. aeruginosa* isolates that were randomly chosen from the isolate's collection.

1.1.1. FastPrep-24 Instrument coupled to QuickGene DNA tissue kit S

The first extraction method that we tested was the QuickGene DNA tissue kit S coupled to FastPrep lysis. This method has been proved to be very efficient for DNA extraction of *Staphylococcus aureus* (Price et al. 2014), thus we decided to test it in *P. aeruginosa* extraction. Initially, bacterial biomass on half blood agar plate that was incubated overnight was resuspended in 0.9% NaCl solution, as in the protocol used for *S. aureus* DNA extraction. In parallel, 5 ml of overnight liquid cultures were also tested for DNA extraction, in order to standardize the bacterial count and therefore have the similar DNA quantity. In the latter case, bacterial pellets were washed with LB containing 10% glycerol to reduce the amount of extracellular polysaccharides that could interfere with the extraction protocol. Extracted DNA from the two methods was processed using the standard DDSL protocol and the corresponding fingerprint patterns are shown in Figure 8. We observed that when the starting material was taken from agar plates a generally lower band quality was observed, and samples showed a higher heterogeneity in DNA quantities. This resulted in bad migration with important "smearing" (Lanes 1-8, Figure 8).

On the other hand, using liquid cultures, the method showed similar band patterns between samples (Lanes 9-16, Figure 8).

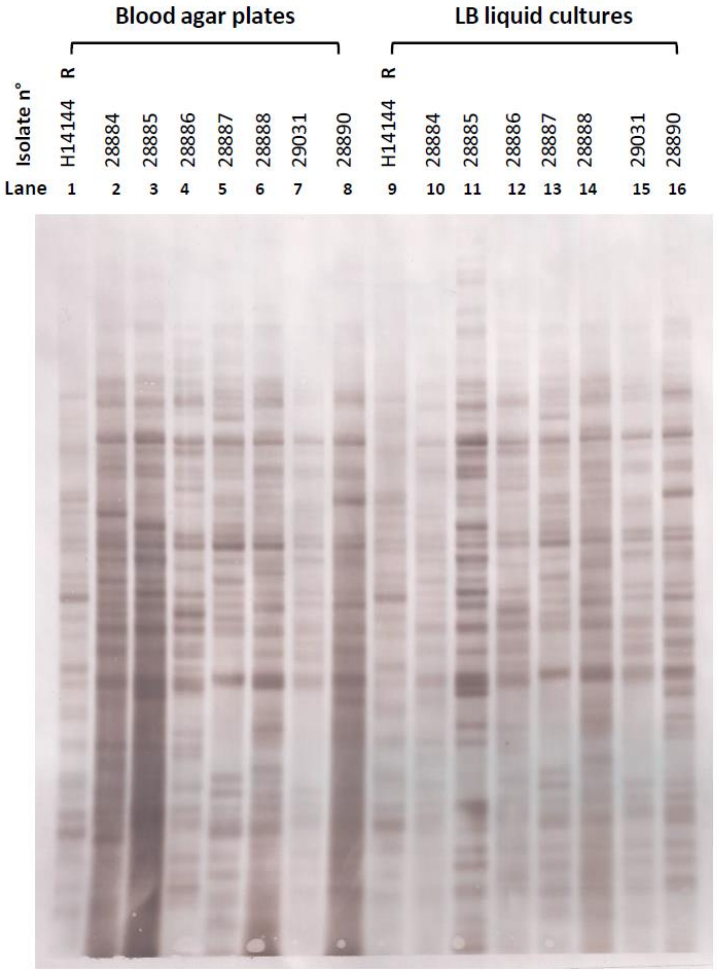


Figure 8. DDSL fingerprint of *P. aeruginosa* isolates DNA extracted with FastPrep-24 Instrument coupled to QuickGene DNA tissue kit S. Lanes 1-8: extraction from blood agar cultures; lanes 9-16: extraction from LB liquid cultures. Lanes 1 and 9 (R) correspond to the *Pseudomonas* subsp. reference strain H 14144.

These DDSL fingerprint patterns were of insufficient quality for analysis with the BioNumerics software. Compared to previous DDSL fingerprint patterns (Terletskiy et al. 2008), bands of high molecular weight were not present, indicating that the extraction method is not optimal for this application. Silica beads may not have only disrupted the bacterial cell wall, but also had a major effect on the DNA molecules size. The poor reproducibility of the results, especially for bands of higher molecular weight, seem to confirm this hypothesis, as larger DNA fragments have a higher probability of being

disrupted by silica beads. Moreover, mechanically induced DNA breaks, coupled to the subsequent enzymatic restriction can create additional artificial bands, giving a smearing-like pattern that might impair the result. In order to avoid this problem, DNA extraction was repeated without the silica beads-beating step. However, with this modification, the protocol could not be carried out, because during the first pressurization, the samples did not pass through the cartridge due to their high viscosity. Given the unsatisfactory results, the QuickGene DNA tissue kit S coupled to FastPrep lysis extraction method was abandoned.

1.1.2. Phenol-chloroform extraction method and GenElute bacterial genomic DNA Kit

Since the QuickGene DNA tissue kit S did not appear to be suitable for the DDSL method, two additional DNA extraction methods were tested and compared: the phenol-chloroform extraction method described by Terletskiy *et al*. and the commercial GenElute bacterial genomic DNA Kit. In addition, three different volumes of extracted DNA, 2 µL, 4µL and 8µL, were tested. The results are shown in Figure 9. Bands of samples extracted with phenol-chloroform were less defined and more diffused compared to those extracted with the GenElute bacterial genomic DNA Kit. Variation of the DNA volume did not improve the results with the phenol-chloroform method. On the contrary, GenElute bacterial genomic DNA Kit extraction method produced a good digestion profile when 8 µL of DNA were used, making it a good candidate for our purpose. However, due to problems with the availability of GenElute bacterial genomic DNA Kit, we were obliged to find another suitable extraction method.

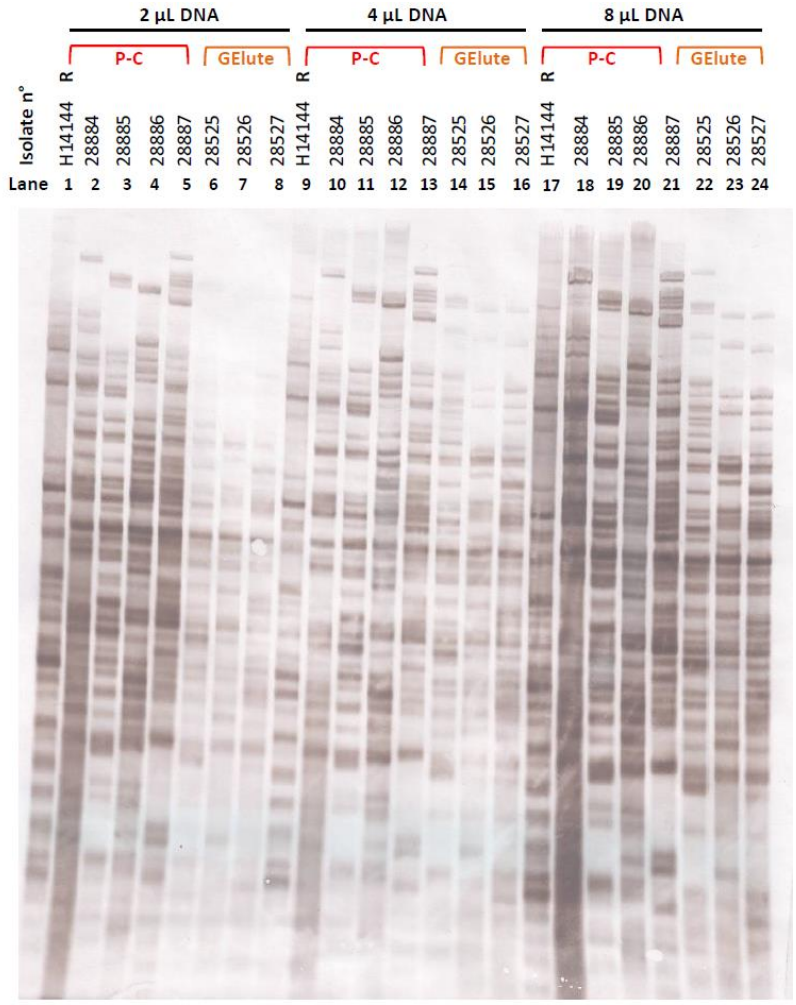


Figure 9. DDSL fingerprint of seven *P. aeruginosa* isolates DNA extracted either with phenol-chloroform or with GenElute bacterial genomic DNA Kit. Different DNA volumes were used: 2 µL, 4 µL and 8 µL. Phenol-chloroform (P-C, in red) was used for the extraction of the first five strains, and GenElute bacterial genomic DNA Kit (GElute, in orange) for the next three strains. Lanes 1, 9 and 17 (R) correspond to the *Pseudomonas* subsp. reference strain H 14144.

1.1.3. Wizard Genomic DNA Purification Kit and GenElute bacterial genomic DNA Kit

The Wizard Genomic DNA Purification Kit was tested as an alternative to the GenElute bacterial genomic DNA kit. Comparison of the results of the two kits is shown in Figure 10.

Figure 10. Comparison of the DDSL fingerprint of *P. aeruginosa* isolates using two different DNA extraction methods: Wizard Genomic DNA Purification Kit and GenElute bacterial genomic DNA Kit. Lanes 1-6 correspondent to (Wizard) samples extracted with Wizard Genomic DNA Purification Kit, and lanes 1-6 correspondent to (GenElute) samples extracted GenElute bacterial genomic DNA Kit (lanes 7-12).

Samples processed with the Wizard Genomic DNA Purification Kit seemed to have problems with bands of high molecular weight, as pseudo-bands were present, probably due to DNA shearing. Given the results of higher quality obtained with the GenElute bacterial genomic DNA Kit when compared to the other methods, we decided to wait for its availability for further testing.

1.2. Optimization of the DDSL protocol

Once the DNA extraction method was set up, DDSL protocol was performed on the first twenty isolates (Figure 11). Despite having genomic DNA of good quality, the results showed a low resolution of bands of high and low molecular weight. In order to correct

this technical problem, we decided to further optimize the DDSL protocol, in particular the electrophoresis and transfer steps.

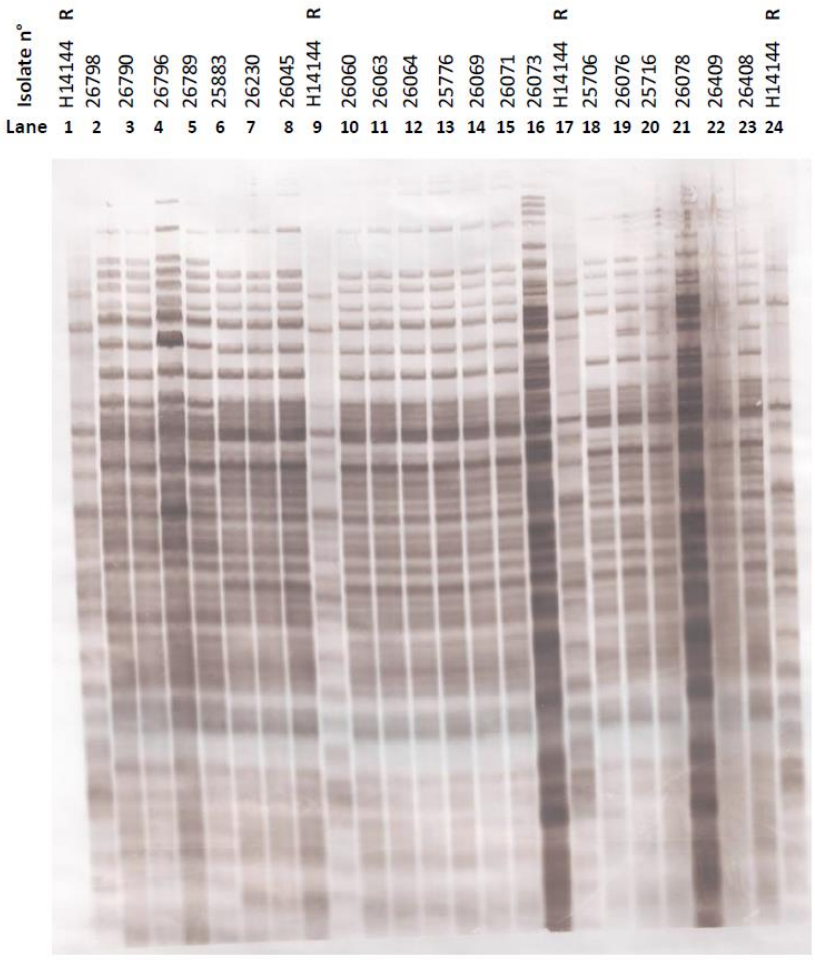


Figure 11. DDSL fingerprint of the first twenty *P. aeruginosa* isolates from cluster 1_18. Lanes 1, 9, 17, and 24 (R) correspond to the *Pseudomonas* subsp. reference strain H 14144.

1.2.1. Electrophoresis temperature

Separation of DNA fragments in agarose gels by electrophoresis is a critical step in the DDSL protocol. In order to avoid diffusion of the DNA fragments due to a high temperature, electrophoresis conditions were changed by reducing the temperature of migration from 30 °C to 14 °C, and consequently increasing the migration time to 5 hours and 10 minutes. The result of this adjustment is shown in Figure 12.

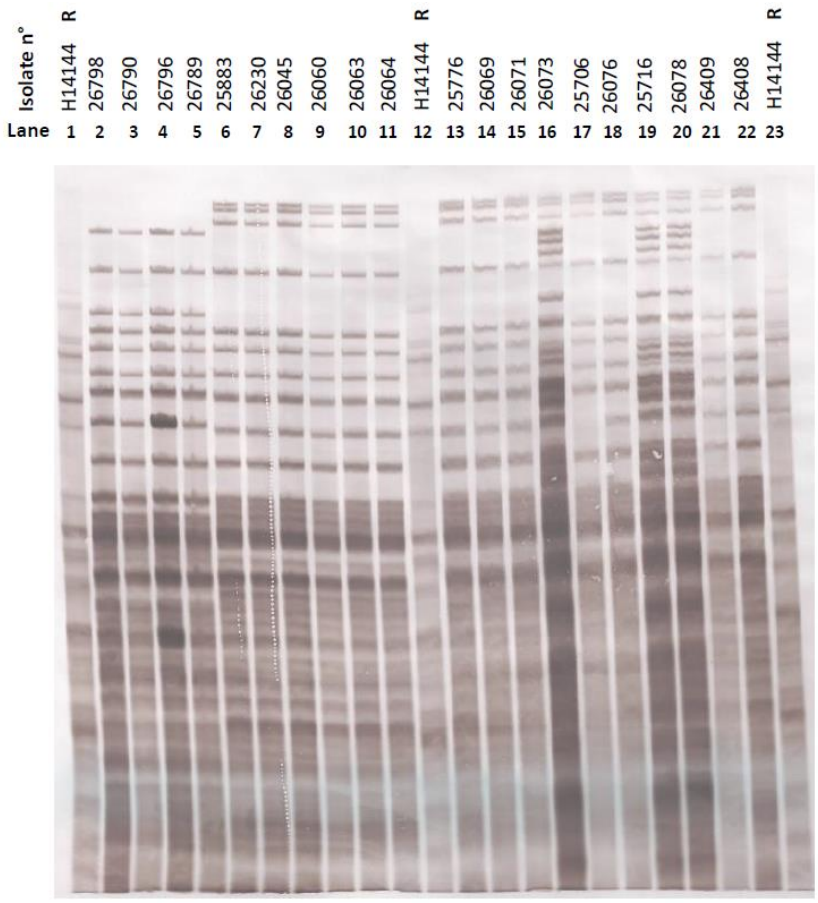


Figure 12. DDSL fingerprint of the first twenty *P. aeruginosa* isolates of cluster 1_18, when electrophoresis was performed at 14 °C, for 5h and 10 minutes. Lanes 1, 12 and 23 correspond to the *Pseudomonas* subsp. reference strain H 14144.

The reduction of the migration temperature had the effect of increasing the resolution of bands of high molecular weight. However, the quality of remaining bands decreased. This was probably due to the increased electrophoresis time needed for a migration at 14 °C, which might favored the diffusion of smaller fragments. For that reason, the migration temperature was kept at 30 °C.

1.2.2. Transfer time and pressure during Southern blot

The transfer of digested DNA fragments from the agarose gel to the membrane is an additional key step of the DDSL procedure. We decided to vary the blotting pressure and time in order to have a better transfer of bands, especially of those of high molecular weight. Thus, blotting pressure was increased to 100 mbar, instead of 40 mbar in the

original protocol. In addition, the blotting time was increased from 40 minutes to 1 hour to favor the transfer of longer DNA fragments. Figure 13 shows that these modifications gave more defined bands compared to previous experiments, as show in Figure 8.

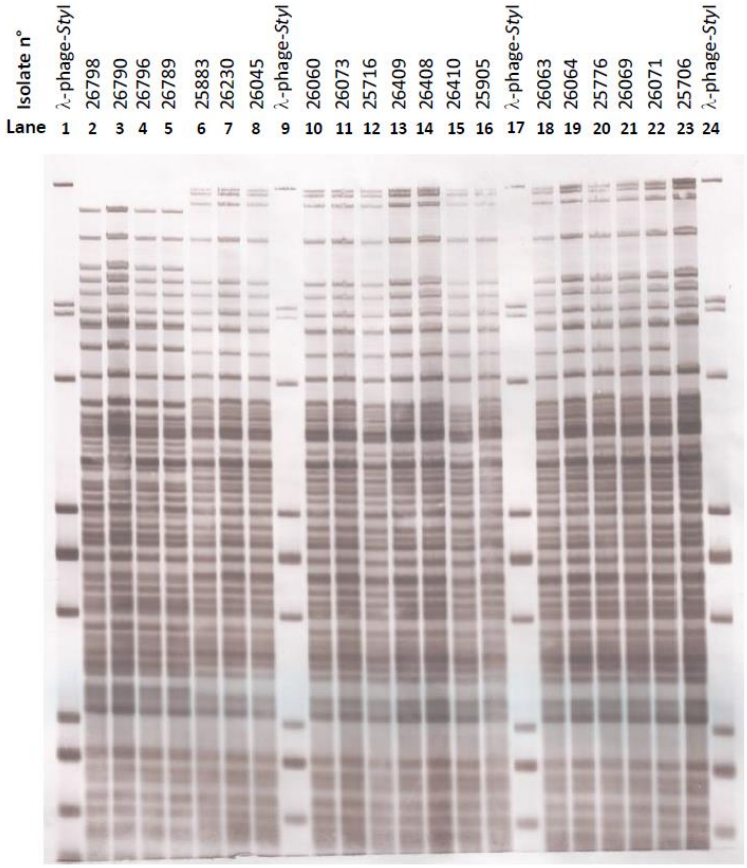


Figure 13. DDSL fingerprint of the first twenty *P. aeruginosa* isolates for cluster 1_18, when transfer time was changed to 1h, and pressure was changed to 100 mbar. Lanes 1, 9, 17 and 24 correspond to the reference marker Lambda phage DNA digested with *Sty*I (λ-phage-*Sty*I).

Overall DDSL fingerprints showed good digestion profiles for *P. aeruginosa* isolates DNA and reference marker. Bands were well defined in almost all profiles, except for the very low part of the membrane. However this part of the gel did not show high variability in band patterns, thus not representing an issue for the analysis of the results.

1.2.3. Selection of the reference marker

The selection of an appropriate reference marker is an important step for the analysis of the results with the BioNumerics software, due to intra- and inter-gel

variations of the migration. Normalization of band fingerprints is done by taking into account the migration of the marker, which allows the comparison of different gels. Therefore, its choice is of utmost importance, and resolution and intensity of its bands should be reproducible. In addition, bands need to be evenly spaced throughout the gel to facilitate the analysis. The original DDSL protocol used the *Pseudomonas* subsp. strain H14144, isolated previously in the hospital for this purpose. However, after many assays, it was not possible to observe a constant pattern with this reference marker. For that reason, we decided to test other strains in order to find a better candidate. A set of ATTC reference strains available in our laboratory collection was tested for this purpose (Figure 14).

Figure 14. DDSL fingerprint of the bacterial ATCC reference strains tested as a molecular-size marker. Lane 1: *Pseudomonas aeruginosa* ATCC 9027; lane 2: *Enterococcus faecalis* ATCC 29212; lane 3: *Escherichia coli* ATCC 10536; lane 4: *Escherichia coli* ATCC 11229; lane 5: *Proteus mirabilis* ATCC 14153; lane 6: *Pseudomonas aeruginosa* ATCC 15442; lane 7: *Pseudomonas aeruginosa La* 2115; lane 8: *Staphylococcus epidermidis* ATCC 12228; lane 9: *Staphylococcus haemolyticus CCM 2737*; lane 10: *Staphylococcus lugdunesis* ATCC 43809; lane 11: *Staphylococcus schleiferi subsp. schleiferi* ATCC 43808; lane 12: *Staphylococcus sciuri subsp. carnaticus* ATCC 700058; lane 13: *Salmonella ser. Braenderup* H9812: lane 14: *Escherichia coli* G4254.

The majority of the candidates did not produce bands of high molecular weight, or showed a “smearing” migration pattern. Nevertheless, even if few strains had acceptable migration pattern and bands of good quality, periodical extraction of the bacterial genomic DNA would increase variability of the reference marker between experiments, as it was the case for strain *P. aeruginosa* H14144.

Another option for the reference marker would be commercially available DNA, which would avoid the DNA extraction step and give more standardized result after enzymatic restriction. Lambda phage DNA was chosen for this purpose, due to its availability and low price. *In silico* digestions of various endonucleases were considered in order to find the most appropriate fingerprint pattern for our study (data not shown). Among the restriction enzymes that were selected, the best candidates were *Hind*III and *Sty*I. Lambda phage DNA was digested with these endonucleases and the DDSL protocol was performed. Several DNA quantities were tested in order to define the optimal concentration. The result is depicted in Figure 15.

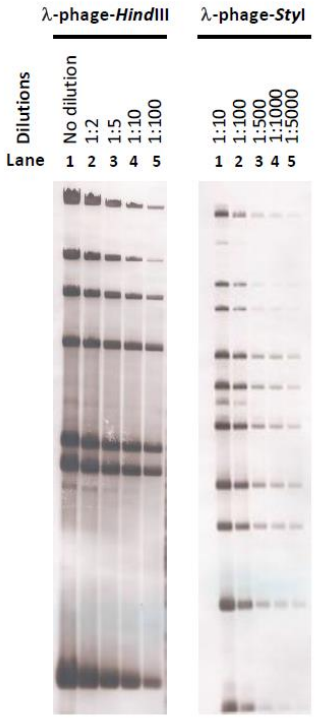


Figure 15. DDSL fingerprint of the Lambda phage DNA digested with the endonucleases *Hind*III and *Sty*I. Several dilutions were tested: (λ-phage-*Hind*III): lane 1-no dilution; lane 2- 1:2; lane 3- 1:5; lane 4- 1:10; lane 5: 1:100; (λ-phage-*Sty*I): lane 1-1:10; lane n 2: 1:100; lane 3- 1:500; lane 4- 1:1000; lane 5: 1:5000.

Both restriction patterns were distributed evenly to cover all the length of the gel. Eventually, *Sty*I was chosen because of the higher number of bands, which would facilitate the analysis of gels.

2. Typing of *P. aeruginosa* isolates with DDSL

2.1. Band-differences comparison

Initially, DDSL fingerprint patterns were analyzed by band-difference comparison method using the auto assign band function in BioNumerics. However, as shown in figure 16, the software incorrectly assigned bands, even after trying different settings. In addition, the quality of the digestion profiles interfered greatly with band recognition, increasing the difficulty of the analysis.

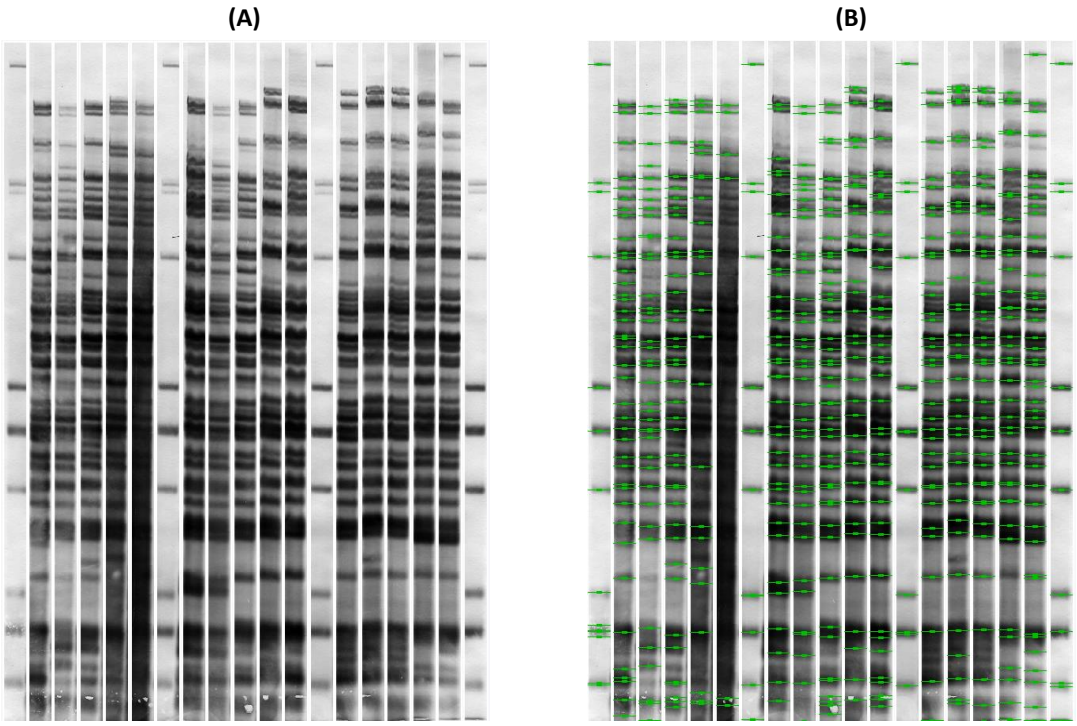


Figure 16. Auto assignment of bands using BioNumerics. (A) Original DDSL fingerprinting profile; the lanes 1, 7, 13 and 19 correspond to the reference marker. (B) DDSL fingerprinting profile showed in (A) to which the auto assignment of bands was performed. Bands identified by the program are represented by green lines.

2.2. Curve-based comparison

Given the scarce results obtained by the auto-assignment of bands function in BioNumerics, a second, curve-based comparison method, was tested on DDSL patterns to verify if an automated analysis was still possible (Figure 17). As a result, isolates were separated in four clusters, corresponding to the four DLST clusters. However, it considered banding patterns as different when, in fact, they were visually undistinguishable. Hence, this method was not adequate for our purpose to further resolve each cluster.

2.3. Visual analysis

Since the BioNumerics software could not perform the correct auto-assignment of bands or the correct identification of similarity values by curve-based comparison, we decided to perform a visual analysis of the results. Isolates originating from one patient were compared and if the fingerprinting results were identical, only one isolate was retained for the final analysis. Fingerprinting patterns of insufficient quality were excluded from the study. This resulted in the selection of 71 isolates out of the initial 152 that were analyzed by the BioNumerics software. A new DDSL type was considered if it differed by one or more bands from the other DDSL types. For each of the four clusters (1_18, 1_21, 6_7 and 28_77), several DDSL pattern types were found. Table 6 shows the number of isolates and number of types found for each DLST cluster. For the analysis, we took in account only the three major DDSL types of each cluster. The remaining types were grouped in the “unique” category, which represents the number of strains belonging to a unique DDSL type.

Table 6. Different DDSL types found for each cluster.

Clusters	**1_18**	**1_21**	**6_7**	**28_77**
N° of isolates*	27	16	15	13
N° of types	5	12	7	11
Type 1	19	3	7	3
Type 2	5	2	2	
Type 3		2	2	
Unique	3	9	4	10

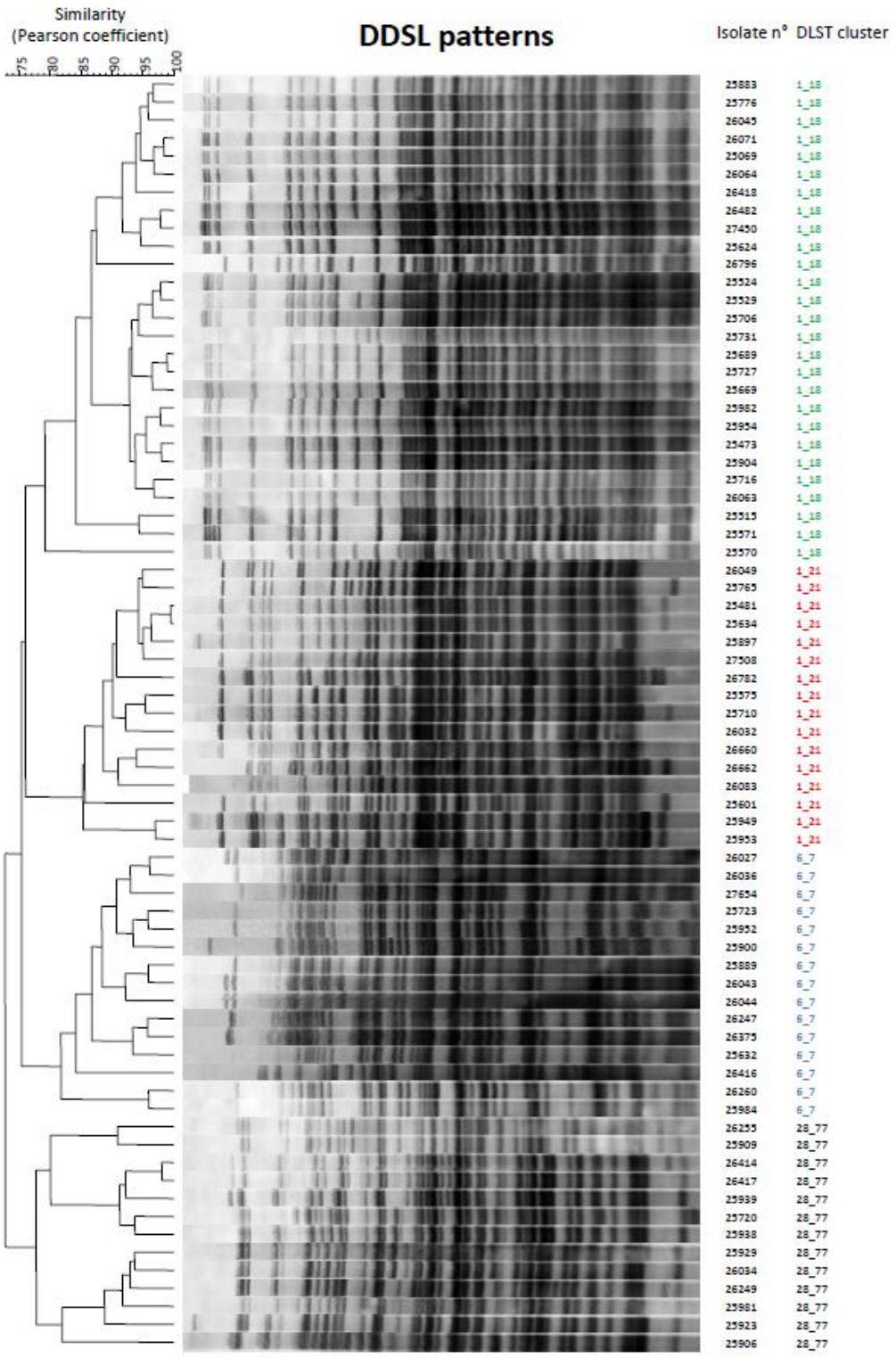


Figure 17. Dendrogram representation of DDSL banding patterns from clusters 1_18, 1_21, 6_7 and 28_77. Similarity values, calculated with the Pearson's similarity coefficient, were used in the construction of a similarity matrix, which was then converted into a dendrogram. For each isolate of each cluster it is shown its respective banding profile and its correlation with other isolates.

For DLST cluster 1_18, only two major DDSL types, type 1 and 2, were found. The majority of the isolates belonged to type 1, while only 3 possessed a unique DDSL type. On the other hand, almost all isolates of DLST cluster 28_77 were of a unique type, with only 3 belonging to DDSL type 1. Lastly, DLST clusters 1_21 and 6_7 harboured isolates from 3 different DDSL types, with DLST cluster 1_21 showing a higher number of unique types when compared to DLST cluster 6_7. In summary, DLST cluster 1_18 accounted for the lowest intra-cluster diversity, whereas DLST clusters 1_21 and 28_77 showed a higher diversity between strains of the same cluster.

3. Epidemiological information

The number of ICU admissions at the University Hospital of Lausanne, from 2010 to 2012, as well as the number of patients with positive samples for *P. aeruginosa*, is shown in Table 7.

Table 7. ICU admissions of patients with *P. aeruginosa* infections during the period of the study (2010 – 2012).

	2010	**2011**	**2012**
Number of ICU admissions (adults)	1910	1990	2025
Number of ICU admissions (children)	335	391	351
Number of ICU admissions (total)	2245	2381	2376
Number of patients with *P. aeruginosa*	100	96	76
Number of patients with *P. aeruginosa*/1000 admissions	44.5	40.3	32.0

Table 8 shows epidemiological data associated to the four DLST cluster. Isolates belonging to DLST cluster 1_18 infected the highest number of patients compared to other clusters. Those patients were mostly admitted due to severe burn injuries, had an average age comprised between 50 and 56 years, and the majority were males. The average length of stay in an ICU was variable between clusters, with patients infected with isolates from DLST cluster 1_21 having the longest length of stay of 102 days. The remaining clusters did not show clear linkage to any specific cause of admission. Lastly, a relatively high mortality was observed for all patients, varying between 20 and 33% of

death occurrence. Cluster 28_77 showed the highest percentage of death during hospitalization (33%).

Table 8. Patients characteristics for each cluster.

	DLST clusters			
	1_18	**1_21**	**6_7**	**28_77**
Number of patients	25	15	14	12
Average age (years)*	51	56	54	50
Males (%)	14 (56%)	11 (73%)	12 (86%)	7 (58%)
Average length of stay in ICU (days)	67	102	32	48
Burn injuries (%)	18 (72%)	0	5 (36%)	0
Death during hospitalization (%)	5 (20%)	3 (20%)	3 (21%)	(33%)

4. Epidemiological links and DDSL types distribution

In order to determine the epidemiological links between patients of the same DLST cluster and/or between patients and environmental isolates, the hospitalization period, the ICU where the hospitalization occurred and the ICUs environmental sampling of *P. aeruginosa* were schematically represented in Annex 1-4. In addition, DDSL types of each isolate belonging to the three major types are shown for each DLST cluster.

4.1. Cluster 1_18 (Annex 1)

Eleven epidemiological links between patients hospitalized in the same ICU, during overlapping periods of time, were found for this cluster: ten in the ICU n° 5 (Burn unit; Annex 1, green) and one in the ICU n° 4 (Annex 1, red). Moreover, two epidemiological links were also found between a patient and an environmental sample, in separate rooms of the burn unit. This environmental sample was retrieved under the shower mattresses present in the hydrotherapy room of the burn unit. In total, 13 epidemiological links were observed for cluster 1_18. Regarding the DDSL banding pattern types for this cluster, Annex 1 shows a distribution of DDSL type 1 throughout all study period. Such distribution supported the probability of patient-to-patient transmission during the outbreak, since several isolates belonging to this type were consistent with the

occurrence of epidemiological links. On the other hand, although DDSL type 2 isolates were also present during the whole period of the study, they could be associated with an epidemiological link only in one case.

4.2. Cluster 1_21 (Annex 2)

A total of four epidemiological links were identified for DLST cluster 1_21, two between patients in the same ICU (first in the ICU n° 5 and the second in ICU n° 2) and two between a patient and an environmental sample (ICU n° 2). In the latter case, the presence of *P. aeruginosa* was detected and retrieved from sink traps. Three major DDSL types were found for cluster 1_21. DDSL types 1 and 2 were distributed through all the study period and were not associated with any epidemiological link. DDSL type 3, in turn, was consistently associated with the presence of epidemiological links in the ICU n° 2.

4.3. Cluster 6_7 (Annex 3)

Only three epidemiological links were identified for DLST cluster 6_7, all between patients in the same unit (ICU n° 4, burn unit), during overlapping periods of time. No epidemiological links between patients and environmental sources were observed. Annex 3 shows the distribution of DDSL type 1 from approximately the middle to the end of the study period, but no epidemiological links were observed during this period. DDSL types 2 and 3 were present at the beginning of the study. It was not found an epidemiological link for isolates of type 2, but type 3 isolates were associated with the occurrence of an epidemiological link.

4.4. Cluster 28_77 (Annex 4)

The last cluster showed only two epidemiological links between patients in the same ICU, one in the ICU n° 4 and the other in the pediatric ICU. Again, absence of epidemiological link between patients and samples collected from the environment was observed. Cluster 28_77 showed only one major DDSL type and the rest of the types were classified as unique. DDSL type 1 was observed during all the period of the study although without being related to any epidemiological link.

V. Discussion

Pseudomonas aeruginosa is one of the most important nosocomial pathogens, responsible for high levels of morbidity and mortality, especially in patients suffering from serious clinical conditions (Campana et al. 2004; Yetkin et al. 2006). Development of epidemiological tracking tools played a crucial role in determining the sources of infection, especially during outbreaks (Basset and Blanc 2014; Miranda et al. 2001; Terletskiy et al. 2008). Due to *P. aeruginosa* diversity and complexity, as well as the variety of niches it can survive in, only studies using powerful molecular typing methods can give insight into the routes of colonization and/or infection (Fothergill et al. 2010; Foxman et al. 2005). The epidemiology of *P. aeruginosa* has been studied with various molecular typing methods, such as PFGE, MLVA, MLST, DLST and DDSL (Tenover et al. 1995; Terletskiy et al. 2008; Turton et al. 2010; Wiehlmann et al. 2007). Compared to other methods, DDSL offers important practical advantages. About 20 isolates can be analyzed at the same time using regular laboratory equipment, without the need for specific instrumentation, as required for PFGE. Cost of the analysis is highly reduced compared to methods using DNA sequencing technology, as in the case of MLVA or MLST. Most importantly, DDSL has been shown to have a high discriminatory power, which allows the resolution of genetic clusters that could not be discriminated using other typing methods (Terletskiy et al. 2008).

1. Technical optimization of the DDSL typing method

A modified DDSL protocol, previously published by Terletskiy *et al.*, was developed in this study. Some modifications were made in order to optimize the quality of fingerprints, and to further reduce the turnaround time and manipulation complexity. The optimization started with the genomic DNA extraction step, where three commercially available kits were tested. GenElute bacterial genomic DNA Kit gave the best results in terms of DNA quality and ease of use. Another modification of the original protocol included the increase of blotting pressure and time to 100 mbar and 1 hour, respectively. The outcome of this modification showed a very well defined DDSL digestion profile, with bands well marked throughout the entire profile. The bands of high molecular weight were well defined and reproducible, suggesting a better transfer from the agarose gel to

the membrane. In the very low part of the membrane, the presence of more diffused bands did not impair the analysis of the results.

In the optimized conditions, DDSL typing was executed within one working day (8h), if DNA extraction was performed the day before. The working time required for this procedure was comparable with those of other PCR-based typing techniques, making it a possible alternative to the latters. In addition, it was possible to analyze 20 strains in parallel for each experiment, at a relatively low cost.

1.1. Selection of DNA molecular size marker

The original protocol for DDSL analysis used a clinical strain as the reference marker for the analysis of the fingerprinting pattern. In this case the DNA isolation step had to be performed at every analysis, increasing the risk of variability of the results, especially for bands at high molecular weight. Strains from different species have been tested to find a better candidate strain, but none of them gave satisfactory results.

In order to standardize this crucial step, we created a marker using commercially available Lambda phage DNA that was digested with *Sty*I endonuclease. By doing this, we were able to have a highly concentrated marker that could be stored at -20 °C and used for all the gels analyzed in this study.

2. Analysis of the DDSL fingerprinting patterns

The fingerprinting profiles obtained with the DDSL method were analyzed using the BioNumerics software. Initially, we decided to compare the band profiles of each isolates and determine the number of band differences between them. However, auto-assignment of band positions by BioNumerics was not possible, as it did not recognize bands that were present or assigned bands that were not present on the gels. Although several parameters were changed and tested, we could not obtain a reliable attribution of bands to the banding profiles. A probable explanation was the quality of the DDSL profiles, which did not always show an optimal resolution of bands. To circumnavigate this problem, bands could have been assigned manually in BioNumerics software, but this

exercise would have greatly increased the complexity and time of analysis, and it was not carried out.

In addition to the auto-assignment of bands position, the curve-based comparison of DDSL patterns was also performed. Relations between isolates assigned by BioNumerics using the Pearson coefficient were not in agreement with a visual comparison of DDSL fingerprints. For instance, DLST cluster 1_18 appeared to have the highest similarity between strains, as most of the digestion profiles were identical. However, the software incorrectly decreased the similarity of fingerprint patterns within this cluster, by taking in account artefacts present on the gels. The same problem could be identified for the other clusters, where no concordance between visual and software analysis was observed. Thus, DDSL fingerprints analyzed with curve-based coefficients of similarity did not allow correct differentiation of fingerprints within clusters. One reason was probably due to the fact that the influence of dissimilar bands was not high enough to compensate the variation in intensity and migration of identical bands. Variability within migration was observed when samples differed in DNA concentration. DNA quantification could be standardized in order to obtain band profiles similar in bands intensity, but would greatly increase the time and complexity of the analysis. In addition, a higher concentration of DNA increased the background, making the detection of bands not reproducible. DNA fragments separation by capillary electrophoresis would avoid this problem, but was found to be technically not possible (Terletskiy et al. 2008).

A solution to these technical problems was the visual comparison between DDSL band patterns, which helped us to cluster isolates correctly and resolve the four DLST groups. DLST cluster 1_18 showed the highest level of similarity between strains, as opposed to DLST cluster 28_77, which possessed the lowest number of identical DDSL profiles. Quantitative comparison was again not possible, since differentiation only resulted in the aggregation of strains in different types. It was not possible to determine the number of different bands observed between each profile.

3. Patients epidemiological data

Incidence of this bacterium decreased from 2010 to 2012, suggesting that infection control measures that were applied in the ICUs reduced the impact of *P. aeruginosa* transmissions. General epidemiological description of the patients belonging to the four DLST clusters showed that the age of colonized/infected persons with this bacterium was higher than 50 years. This was concordant with the assumption that *P. aeruginosa* affects mostly immunocompromised patients and elder patients tend to be more debilitated and prone to infection (Blanc 2007, Hakki 2007). The observed levels of mortality were high (20-30%), which was in concordance with other published studies (Hakki et al. 2007; Kim et al. 2014). Interestingly, the majority of the patients carrying this bacterium were males (56 - 86%, depending on the cluster). DLST cluster 1_18 comprised the highest number of patients and the majority of them were hospitalized due to burn injuries (72%). Additionally, the average length of ICU stay was relatively long (67 days). The occurrence of such large cluster suggests the contribution of a persisting source of infection, probably from an environmental reservoir. *Pseudomonas aeruginosa* isolates belonging to DLST cluster 1_18 were retrieved from samples collected from shower mattresses present in the burn unit hydrotherapy room, further supporting the assumption of an environmental source of infection. Environmental samples belonging to this DLST type were also found in sink traps present in the burn unit. Thus, the occurrence of an outbreak of *P. aeruginosa* belonging to DLST cluster 1_18 was most probably due to infringement of good practices during the disinfection procedures of shower mattresses and trolleys, which remained wet after disinfection, as well as the contamination of sink traps. However, patient to patient transmission could also have occurred subsequently, as demonstrated by the fact that some patients were not taken into the shower room. Following the implementation of infection control measures, the number of patients with *P. aeruginosa* associated to this cluster was found only once in the following years of surveillance. Cluster 1_21 involved fewer patients and was not related to the burn unit. The fact that an environmental sample recovered from sink traps was collected in the same room where two patients were hospitalized (ICU n° 2) suggested the sink trap might be the reservoir. Clusters 6_7 and 28_77 showed no epidemiological links between environment and

patients. Only a few cases of patient-to-patient transmissions could be suspected for both clusters.

Although all of the data suggested a major role of environmental sources, it was unknown whether the outbreak originated from the environment or if it was associated to a contaminated patient, as the environmental sampling information was only available since 2012. A more frequent and regular sampling throughout the study period would have been necessary to further characterize the source of infection.

4. Comparison between epidemiological and typing data

Bacteria reproduce by binary fission and mutations are expected to accumulate over time. Thus, two isolates that are distantly related in time should show a higher genetic variation than two isolates that are recovered in a small period of time. Thus, in theory, the majority of undistinguishable isolates by DDSL should be epidemiologically linked, while dissimilar DDSL isolates should be unlinked. This means that in most of the cases where patients were hospitalized in the same ICU, for overlapping periods of time, sampled isolates should belonged to the same DDSL type. This was the case with the DLST cluster 1_18, in which the predominant DDSL type 1 was distributed throughout the whole period of the study and was correspondent to a high number of epidemiological links. However, the same did not happen with cluster 28_77 in which the presence of undistinguishable DDSL types showed few epidemiological links. Consequently, the DDSL method did not provide enough quantitative information on the accumulation of genetic mutation over time that could link these mutations to epidemiological data. Whole Genome Sequencing (WGS), which allows the analysis of single nucleotide polymorphisms (SNPs) on the whole bacterial genomes, should enable such epidemiological tracking. In 2013, Snyder *et al*. used WGS of five linked *P. aeruginosa* isolates, collected during a six-year outbreak to discover several SNPs that may be responsible for phenotypic differences between the isolates, and even be crucial for it survival in hospital settings (Snyder et al. 2013). Such technique is more powerful compared to other molecular methods and appears very promising for analysis of epidemiological studies.

VI. Conclusions and future perspectives

Pseudomonas aeruginosa is one of the most important and complex pathogens acquired in hospital settings. It causes a variety of clinical outcomes, especially in immunocompromised patients, contributing to high morbidity and mortality levels. The ICU incidence of this bacterium and the number of nosocomial infections it causes continues to increase, even though infection control measures are implemented. In order to improve the early detection of *P. aeruginosa* contamination during an outbreak episode, new fast and efficient diagnostic strategies need to be developed. Molecular typing has been proved to be very efficient in resolving cases of possible transmission between patients, or between patients and the environment.

In this thesis, a new approach in the study of *P. aeruginosa* epidemiology was explored using DDSL, which was expected to be a good complementary method to resolve DLST clusters due to its higher discriminatory power. After several steps of optimization, DDSL protocol resulted in relatively clear and defined digestion profiles. The DDSL procedure could be performed within one working day (8 hours) allowing analysis of 20 strains in parallel, with relatively low cost. Several attempts to quantitatively analyse the DDSL fingerprinting patterns using the BioNumerics software, either by auto-assignment of bands, or by curve-based comparison, were performed. However, the results were not satisfactory due to several issues. Quality of the digestion profiles was the major problem, as some of the bands were poorly defined, and the presence of a strong background on the gel interfered with the analysis. Eventually, visual comparison between DDSL banding patterns enabled the grouping of strains in different DDSL types for every DLST cluster. This analysis was therefore qualitative, since it was not possible to quantify bands differences between DDSL profiles. Nevertheless, this approach permitted to obtain important information about possible sources of infections and transmissions chains. However, it is a subjective, time consuming and technically complex technique that is not suitable for a diagnostic laboratory.

Double locus sequence typing is an efficient and good typing method to group isolates in genetic clusters, but a more discriminatory molecular typing technique should be developed and used in order to give more insights on *P. aeruginosa* epidemiology during the occurrence of ICU outbreaks. Whole genome sequencing could be a good

alternative to molecular methods currently used; but it is still very expensive and analysis of results is not yet standardize.

VII. References

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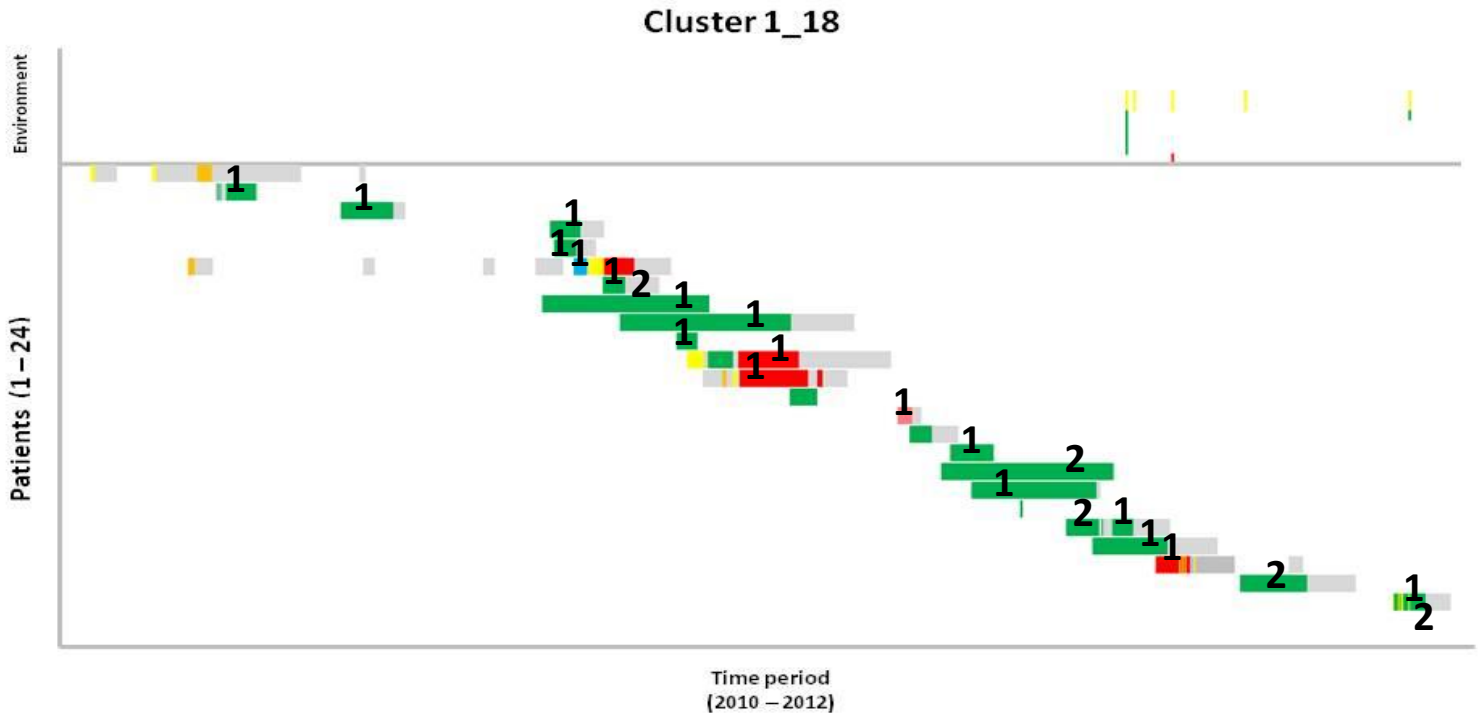
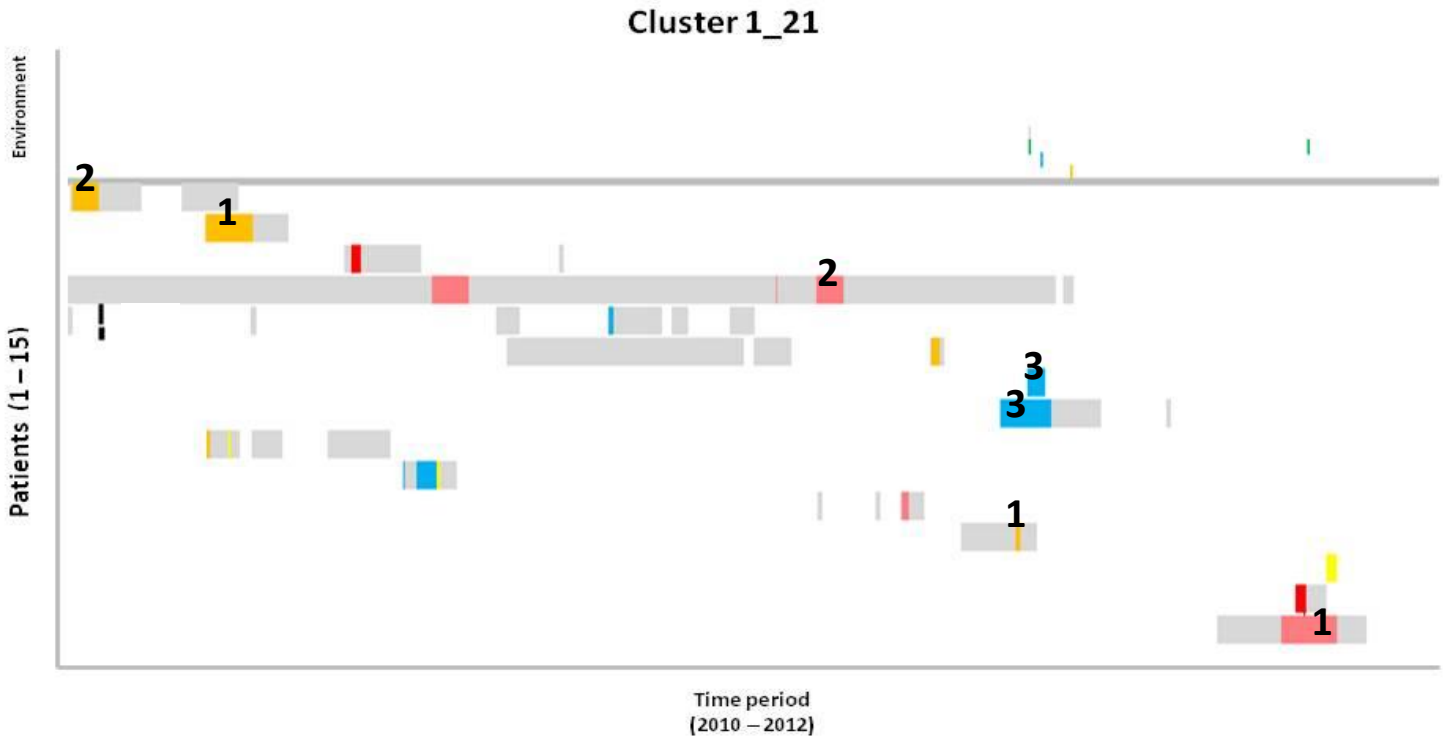
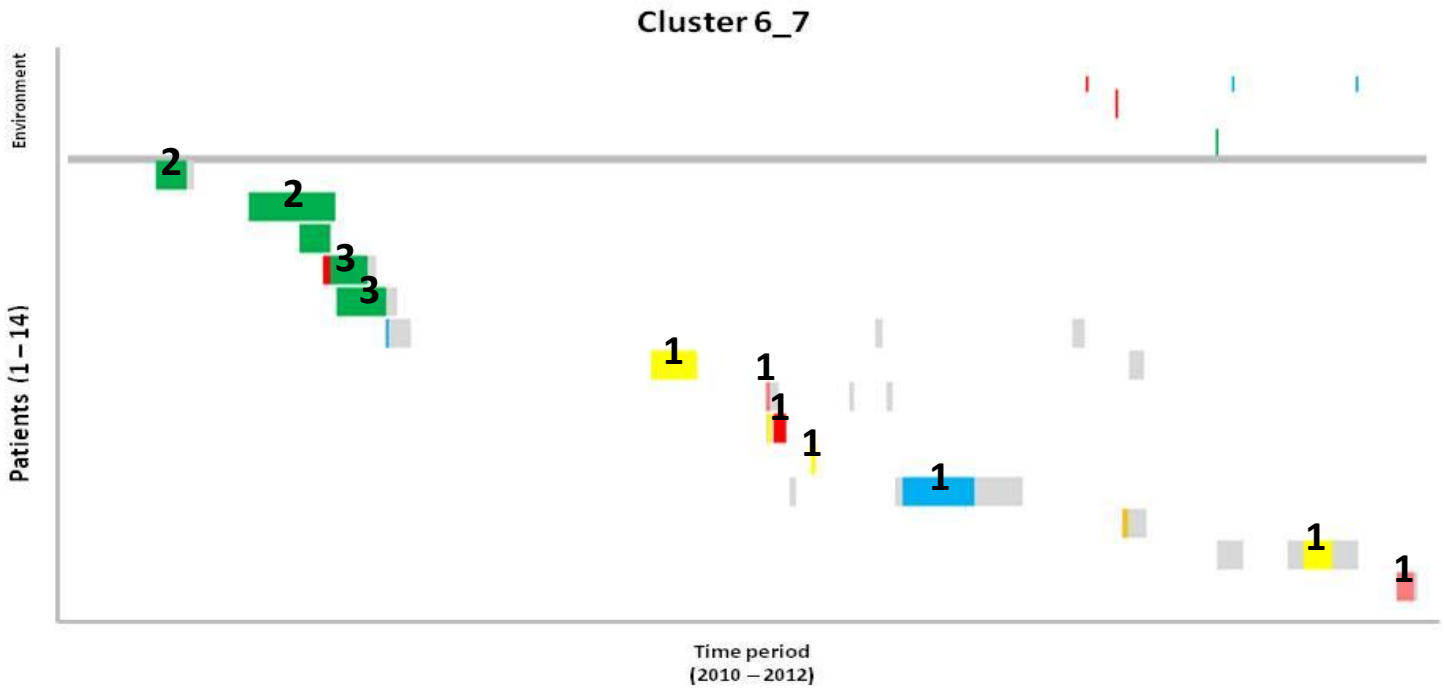
VIII. Annexes



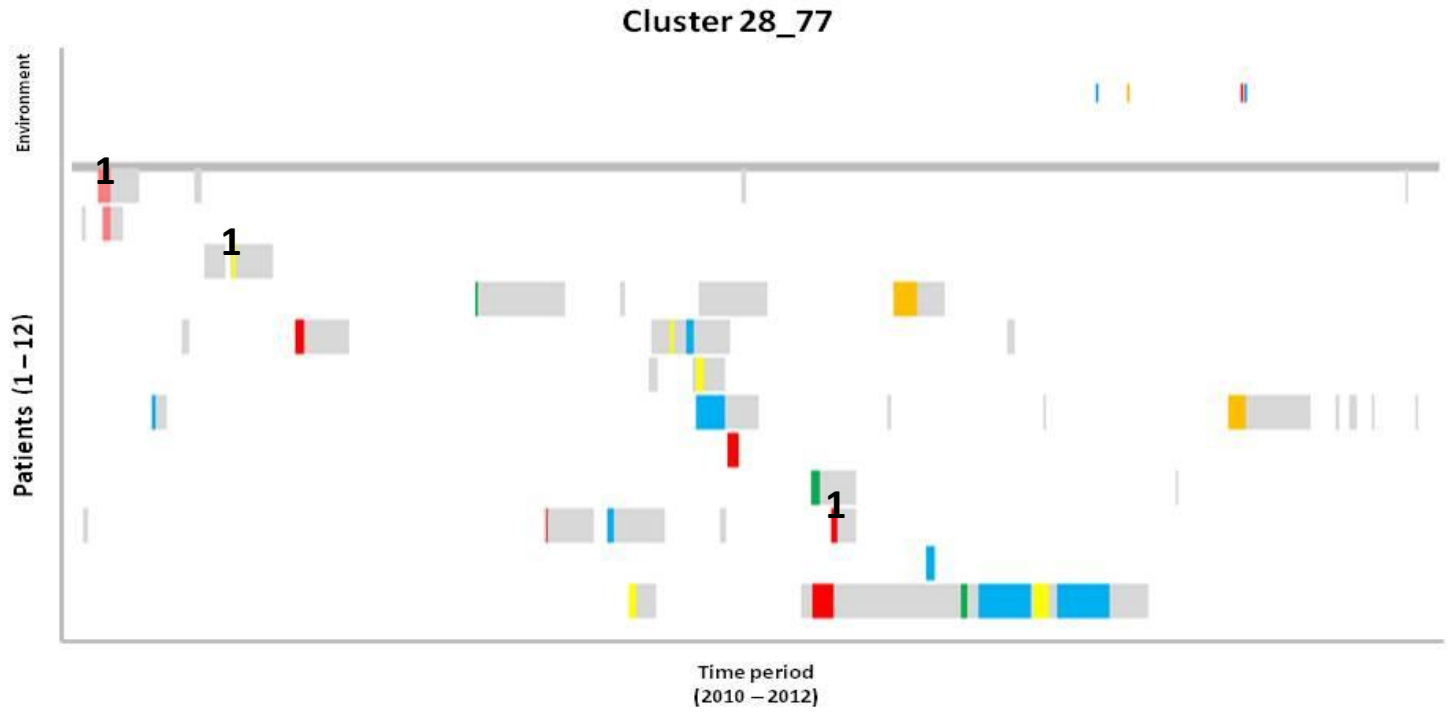
Annex 1. Representation of the hospitalization period, ICU where the hospitalization occurred and ICUs environmental sampling of P. aeruginosa for cluster 1_18. Environmental sampling information is present in the upper quadrant, and patients' epidemiological information is present in the quadrant below. Units where environmental samples were retrieved and patients were hospitalized, are differentiated by colors. ICU 1: yellow; ICU 2: blue; ICU 3: green (burn unit); ICU 4: red; ICU 5: darker yellow; pediatric ICU: pink; hospitalization in non-ICU units: grey. DDSL major types are also represented.



Annex 2. Representation of the hospitalization period, ICU where the hospitalization occurred and ICUs environmental sampling of P. aeruginosa for cluster 1_21. Check Figure 1 (Annex 1) legend for further explanations.



Annex 3. Representation of the hospitalization period, ICU where the hospitalization occurred and ICUs environmental sampling of P. aeruginosa for cluster 6_7. Check Figure 1 (Annex 1) legend for further explanations.



Annex 4. Representation of the hospitalization period, ICU where the hospitalization occurred and ICUs environmental sampling of P. aeruginosa for cluster 28_77. Check Figure 1 (Annex 1) legend for further explanations.